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UNIVERSITY OF ALBERTA

"Estradiol modulation of PMA- and ionomycin-stimulated gonadotropin secretion from anterior pituitaries of male rats."

BY

Nabil Wassili Fahmy

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

IN

Endocrinology

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

FALL, 1990

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DEDICATION

This work is dedicated to my wife Hala and my daughter Carol for their endurance and support during the progress of the research.

ABSTRACT

Phorbol 12-myristate, 13-acetate (PMA) and ionomycin were used as probes to (i) delineate the roles and contributions of the calcium messenger system during gonadotropin secretion from pituitaries of male rats, and (ii) investigate potential effects of estradiol on this system.

Quartered anterior pituitaries obtained from diestrous II female, male and gonadectomized (72 h) + estradiol(E_2)-treated (24 h) male or female rats were placed in perifusion chambers at 37°C. The pituitary tissue blocks were perifused with Krebs Improved Ringer I bicarbonate medium in the presence or absence of various secretagogues. Sequential effluent samples were collected every 10 min for 6 h. LH and FSH concentrations in the samples were later estimated by radioimmunoassay.

Infusions of PMA stimulated gonadotropin secretion from pituitaries of diestrous II, ovariectomized + estradiol-treated, and orchidectomized + estradiol-treated rats, by protein synthesis-dependent mechanisms as determined by treatment with cycloheximide. In contrast, pituitaries from intact males, orchidectomized males or ovariectomized females were unresponsive to PMA. Unlike PMA, ionomycin stimulated gonadotropin secretion from pituitaries of intact, castrated and castrated + estradiol-treated males, with estradiol causing a protein synthesis-dependent enhanced response. The responses to simultaneous infusions of PMA and ionomycin demonstrated synergistic interactions from pituitaries of intact or castrated males, but not from pituitaries of castrated + E_2-treated males.

Gonadotropin releasing hormone (GnRH) stimulated a low, rapid,

extracellular calcium-independent component of gonadotropin secretion from pituitaries of intact, castrated and castrated + E_2-treated males. Interestingly, estradiol enhanced the GnRH-stimulated gonadotropin secretion by inducing an additional delayed (about 70 min) protein synthesis-dependent component. While PMA in calcium-free medium was ineffective in stimulating gonadotropin secretion from pituitaries of intact or castrated males, the phorbol ester stimulated a delayed (about 60 min) protein synthesis-dependent component from pituitaries of castrated + E_2-treated males.

These results indicate that estradiol can enhance gonadotropin secretion induced by ionomycin and/or PMA from anterior pituitaries of male rats. The estradiol-enhanced responses are dependent on de novo protein synthesis, and appear to result in GnRH-stimulated composite secretory responses in the absence of extracellular calcium. Finally, the effects of estradiol on PMA-stimulated secretion in female pituitaries, form at least one basis for the estradiol-induced increased responsiveness of female gonadotrophs to GnRH.

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LIST OF ABBREVIATIONS USED IN THE TEXT, FIGURES AND TABLES:

AA	: Arachidonic acid.
ACTH	: Adrenocorticotrophic hormone.
ARGG	: Antirabbit gamma globulin.
Arg	: Arginine amino acid.
BSA	: Bovine serum albumin.
cAMP	: Cyclic adenosine 3',5'-monophosphate.
cDNA	: Complementary deoxyribonucleic acid.
cGMP	: Cyclic guanosine monophosphate.
CM	: Calmodulin.
CMF-BSA	: Calcium-magnesium-free medium with bovine serum albumin added.
cpm	: counts per minute.
DAG	: Diacylglycerol.
D I	: Diestrus I stage of the female estrous cycle, determined by daily vaginal cytology.
D II	: Diestrus II stage of the female estrous cycle, determined by daily vaginal cytology.
DMSO	: Dimethyl sulphoxide.
DNase	: Deoxyribonuclease enzyme.
E_2	: 17 beta estradiol benzoate.
EGTA	: Ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

FSH : Follicle-stimulating hormone.

GDP : Guanosine diphosphate.

GH : Growth hormone.

Glu : Glutamic acid.

Gly : Glycine amino acid.

GnRH : Gonadotropin-releasing hormone.

G-protein : Guanine nucleotide binding protein.

Gpp(NH)p : 5'-guanylyl imidodiphosphate.

5-HETE : 5-hydroxy eicosatetraenoic acid.

15-HETE : 15-hydroxy eicosatetraenoic acid.

His : Histidine amino acid.

h : Hour.

IP_3 : Inositol 1,4,5-trisphosphate.

KCl : Potassium chloride.

KIRB : Kreb's improved Ringer I bicarbonate.

LC_4 : Leucotriene C_4.

Leu : Leucine amino acid.

LH : Luteinizing hormone.

M : Molar.

mBq : Millibecquerel.

MgSO4 : Magnesium sulphate.

n : number of experiments.

NIADDK : National Institute of Arthritis, Diabetes, Digestive and Kidney disease, U.S.A.

OVX : Ovariectomized rat.

$OVX+E_2$	: Ovariectomized (72 h), estradiol benzoate-implanted (24 h) rat.
P	: Progesterone.
PBS	: Phosphate-Buffered Saline.
PG	: Prostaglandins
PI	: Phosphoinositides.
PKC	: Protein kinase C, calcium-activated, phospholipid-dependent protein kinase.
PMA	: Phorbol,12-myristate,13-acetate.
PRL	: Prolactin.
Pro	: Proline amino acid.
PS	: Phosphatidyl serine.
pyro	: Pyrrolidone group.
SBTI	: Soyabean trypsin inhibitor.
s.c.	: Subcutaneously.
SEM	: Standard error of the mean.
Ser	: Serine amino acid.
T	: Testosterone.
Trp	: Tryptophan amino acid.
TSH	: Thyroid stimulating hormone.
Tyr	: Tyrosine amino acid.

CHAPTER I

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a decapeptide synthesized by cell bodies of neurons which form the nuclei of the medial basal hypothalamus in mammals (Okon and Koch, 1976). GnRH is concentrated in secretory granules which are carried by axonal flow in the fibres of the tuberoinfundibular tract to the median eminence area, and are stored in the axonal terminals. These axonal terminals are closely applied to capillary blood vessels which form the primary capillary bed of the hypothalamic hypophyseal portal system (Niemineva, 1950; Raiha and Hjeet, 1957; Thliveris and Currie, 1980; Matwijiw et al., 1989).

In the mammalian female, the secretion of GnRH into the portal vessels is controlled by two hypothalamic centres, a tonic center in the medial basal hypothalamus (mainly the area of the arcuate nucleus), and a cyclic center in the anterior hypothalamus (mainly the preoptic area) (Fink, 1988; Kalra and Kalra, 1983; Goodman, 1978). The cyclic center regulates the pre-ovulatory surge of GnRH secretion, while the tonic center regulates the low, tonic GnRH secretion during the remainder of the reproductive cycle. In males, the secretion of GnRH into the hypothalamic hypophyseal portal circulation is regulated by a tonic center alone (Fink, 1988; Kalra and Kalra, 1983; Goodman, 1978). Once secreted, GnRH is transported by the portal vessels from the hypothalamus to the anterior pituitary gland where it regulates the cellular activities of gonadotrophs. These activities involve the stimulation of protein

synthesis-dependent and -independent mechanisms associated with the biosynthesis and secretion of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) (Knobil, 1980; Levine and Duffy, 1988; Crowley et al., 1986; Vogel et al., 1986). The alteration of cellular functions by GnRH is initiated by the binding of the hypothalamic decapeptide to specific protein receptors located on the plasma membranes of the gonadotrophs (Marshall et al., 1980; Conn et al., 1981a; Clayton and Catt, 1981 a&b). The ensuing formation of GnRH-receptor complexes results in an increase in the intracellular concentrations of various mediators or second messengers of GnRH. To date, calcium, diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP_3), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and arachidonic acid (AA) and its metabolites have all been implicated as mediators of the actions of GnRH (Conn et al., 1981a; Catt et al., 1983; 1985; Conn, 1986). Complex interactions between the second messengers culminate in increased protein synthesis (including the biosynthesis of LH and FSH), and an increased rate of secretion of these hormones into the systemic circulation.

The gonadotropins regulate various aspects of gonadal function, associated with gametogenesis and steroidogenesis. In females, these include the stimulation of proteogenesis, folliculogenesis, ovulation, corpus luteum formation and steroidogenesis (Coulson et al., 1972; Speroff, et al., 1983). The predominant steroids elaborated are estradiol (E_2) and progesterone (P). In males, LH and FSH stimulate proteogenesis, spermatogenesis and steroidogenesis [predominantly

testosterone (T)] (Burger et al., 1972; Speroff et al., 1983).

The secretory products of the gonads (steroids and peptides) exert a regulatory effect on the secretion of GnRH and the gonadotropins at the levels of the hypothalamus and/or the anterior pituitary (Fink and Pickering, 1980). In males, androgens exert a negative feedback effect (inhibiting GnRH and LH/FSH secretion), whereas, in females E_2 exerts a positive feedback effect during the pre-ovulatory phase of the reproductive cycle (E_2 increases GnRH secretion and the responsiveness of gonadotrophs to GnRH) (Fink and Pickering, 1980). During the post-ovulatory stages of the cycle, E_2 and P exert a negative feedback effect on GnRH and gonadotropin secretion (Mahesh et al., 1972). Thus, the hypothalamus, the anterior pituitary and the gonads form a hormonally interconnected system where each organ regulates the functions of the others by its endocrine secretions.

The present study was initially designed to investigate the roles of calcium as a mediator of gonadotropin secretion from male anterior pituitaries. This objective was later modified to study the modulation of the roles of calcium by estradiol. Consequently, the literature review will focus on the mechanism of action of GnRH, with an emphasis on the roles of calcium and the effects of estradiol.

A. LITERATURE REVIEW

Chemistry of GnRH.

GnRH is a decapeptide (10 amino acid residues) with a pyrrolidone group at the amino terminal, and an amide group at the carboxy terminal

(Schally et al., 1971). It has the following primary structure:

Pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-amide.

Least energy configuration considerations suggest that the termini of the molecule are in close proximity (Momany, 1976). This configuration is stabilized by the formation of a hydrogen bond between the pyrrolidone carbonyl group on residue 1, and the glycinamide group on residue 10 (Nikolics et al., 1977; Coy et al., 1979a&b). The physiological significance of this conformation lies in the fact that the terminal residues, pyro-Glu and Gly-amide are involved in the formation of a region which binds to the GnRH receptor (Coy et al., 1979a&b). This is best illustrated by the observation that modifications of either residue (one or ten) dramatically change the affinity of the peptide for its plasma membrane receptor (Coy et al., 1979a&b).

In addition to having a site which is important in receptor recognition and binding, the GnRH molecule also has a site which is important in the activation of the second messenger systems. The amino terminal residues, especially the first three amino acids are believed to form a domain that is involved in effector activation and production of the intracellular mediators (Conn et al., 1987a). As one would expect, changes to amino acid residues one, two and three result in the formation of GnRH antagonists, compounds which are capable of binding to the GnRH receptors, but are incapable of activating effector mechanisms and hence, gonadotropin secretion (Conn et al., 1987a).

As stated earlier, GnRH is transported from the hypothalamus to the anterior pituitary where it regulates the activities of the gonadotrophs. Consequently, a brief description of the anatomy of the anterior pituitary

will be undertaken prior to the discussion on the mechanism of action of GnRH.

The Anterior Pituitary Gland.

The pituitary gland is a small endocrine organ which lies in the sella turcica at the base of the brain. It consists of a posterior lobe (pars nervosa), an anterior lobe (pars distalis), an intermediate lobe (pars intermedia) and a pituitary stalk (Farquhar et al., 1975; Daniel, 1976).

The anterior lobe of the pituitary gland develops as an outgrowth of epithelial tissue from the primitive pharynx (Rathke's pouch) which meets a down-growth from the base of the brain, the down-growth from the brain being destined to form the infundibular process (posterior lobe) of the pituitary, as well as the neural part of the pituitary stalk (Reid et al., 1972; Gluckman, 1981).

The anterior pituitary is composed of both secretory and non-secretory cells. The non-secretory cells include follicular cells and macrophages plus other connective tissue cells such as fibroblasts. The secretory cells are predominant in the parenchyma of the gland, and are arranged in irregular cords and clumps which are associated with thin-walled blood vessels called sinusoids (Farquhar et al., 1975). It is commonly accepted that the mammalian anterior pituitary is composed of at least five types of secretory cells. These cells have been distinguished by electron microscopy on the basis of differences in the size of the secretory granules, and the structure of subcelluar organelles (Pelletier et al., 1978), or by immunocytochemistry (Nakane, 1970; Farquhar et al., 1975; Phifer et al., 1973). The five cell types and their secretions

are: 1) the somatotrophs which secrete growth hormone (GH); 2) the mammotrophs which secrete prolactin (PRL); 3) the thyrotrophs which secrete thyroid stimulating hormone (TSH); 4) the corticotrophs which secrete adrenocorticotrophic hormone (ACTH); and 5) the gonadotrophs which secrete LH and FSH. Among the gonadotrophs which represent only 5-15% of the anterior pituitary cells, morphologically distinct subtypes have been distinguished by immunocytochemistry. It appears that some gonadotrophs elaborate only LH or FSH, while others secrete both hormones. Childs et al. (1983) have shown that about 60% of the gonadotrophs contain both LH and FSH, while 18% contain LH alone and 22% contain FSH alone.

A predominant effect of GnRH on the gonadotrophs is an increase in the secretion of the gonadotropins. As a result, important parameters which are central to these studies are the characteristics of the secretory profiles of LH and FSH obtained in response to GnRH.

Secretory Profiles of the Gonadotropins.

LH and FSH are normally secreted from the anterior pituitary gland of males and females in a pulsatile fashion in vivo (Yen and Rebar, 1979). Such gonadotropin pulses from the pituitary are thought to be produced by bursts of GnRH from the hypothalamus (Levine et al., 1982; 1985). Changes in the amplitude and frequency of GnRH pulses occur during the different stages of the estrous cycle, as well as during different stages of gestation in rats (Gallo et al., 1987). Such changes in GnRH pulses result in changes in the amounts of gonadotropins secreted.

Repeated administration of identical GnRH pulses to intact, adult,

male rats results in corresponding gonadotropin pulses which are equal in amplitude (Bourne, G.A., unpublished data). On the other hand, repeated adminstration of equal amounts of GnRH pulses to females result in the secretion of progressively larger amounts of gonadotropins (Aiyer et al., 1974; Rommler et al., 1978; Bourne and Baldwin, 1980; Waring and Turgeon, 1980). This phenomenon of augmented responses after an initial exposure to a GnRH pulse is known as the self-priming effect of GnRH (Aiyer et al., 1974; Rommler et al., 1978; Blake, 1978a; Bourne and Baldwin, 1980; Waring and Turgeon, 1980). This effect which is not elicited from pituitaries of male rats, is dependent on estradiol and protein synthesis, and requires a minimum of 30-70 min to develop depending on both the species and the stage of the reproductive cycle (Evans et al., 1984; Pickering and Fink, 1976; Edwardson and Gilbert, 1975).

Continuous infusions of GnRH to pituitaries of male rats result in a slow rise in LH and FSH secretion which reaches a plateau after 2-3 hours (Bourne and Baldwin, 1987b). Additionally, it appears that the secretion from pituitaries of male rats is dependent on protein synthesis (Bourne and Baldwin, 1987b). In contrast to males, continuous infusions of GnRH to pituitaries of female rats result in biphasic responses (Blake, 1978b; Wang et al., 1976; deKoning et al., 1976a&b; Bourne and Baldwin, 1980; 1987a; Baldwin et al., 1983). The response is characterized by an initial, acute, low release rate of LH (referred to as phase I), which is followed approximately 30-70 min later by a second phase of a greatly augmented secretion rate (referred to as phase II). The initial release is independent of de novo protein synthesis, while the second phase is a

protein synthesis-dependent response (Bourne and Baldwin, 1980; De Koning et al., 1976b).

It is interesting that the time-lag required for the manifestation of the biphasic response and the self-priming effect of GnRH, coupled with their mutual requirements for de novo protein synthesis and estradiol, strongly suggest that these two events represent the same basic phenomenon (i.e. both result from similar underlying mechanisms).

B. MECHANISM OF ACTION OF GnRH

GnRH Receptor Binding.

The first step in the activation of gonadotrophs by GnRH is the reversible binding of the decapeptide to specific GnRH receptors on the plasma membrane of gonadotrophs (Marshall et al., 1980; Conn et al., 1981a; Marian et al., 1983; Conn, 1984; Clayton and Catt, 1981a&b, Clayton et al., 1982; Reeves et al., 1980; Perrin et al., 1980). The reversible hormone-receptor interactions are due to the formation of noncovalent bonds between the hormone and the receptor. The noncovalent bonds involve electrostatic interactions (including hydrogen bonding), hydrophobic interactions and van der Waal's forces (Baxter and Funder, 1979). The electrostatic bonds appear to play an important role in receptor recognition, while the hydrophobic interactions drive the binding reaction in a forward direction (Baxter and Funder, 1979).

The binding of GnRH to its plasma membrane receptor, results in the formation of hormone-receptor complexes which cause the receptors to aggregate and concentrate in one area of the cell surface, a process

referred to as patching (Hopkins and Gregory, 1977). This aggregation and patching is temperature-dependent (Hazum , 1981), since at 4°C the ligand-bound, labelled receptors were found to be evenly distributed over the cell surface, but, when the temperature was raised to 37°C, the receptors rapidly aggregated into patches and were internalized into endocytic vesicles. The sequence of receptor binding, patching and internalization terminates in the lysosomal and Golgi organelles, which function as major sites of accumulation of the internalized receptors (Duello et al., 1983).

Although GnRH receptors are internalized, the process of internalization does not appear to be involved in gonadotropin release. Conn and Hazum (1981) demonstrated that removal of GnRH from the extracellular medium of dispersed pituitary cells after considerable internalization had occurred, results in a prompt extinction of the secretory response. In addition, when a GnRH agonist was cross-linked to an agarose matrix (Conn et al., 1981b), the immobilized agonist was capable of evoking LH release from pituitary cell cultures with the same efficacy as the nonimmobilized hormone, thus uncoupling LH release and GnRH internalization.

In addition to initiating the phenomena of aggregation and patching, the formation of GnRH-receptor complexes induces conformational changes in the receptor (Conn, 1986). This in turn results in the GnRH signal being transduced across the plasma membrane. One of the signal transduction mechanisms involves the activation of a group of guanosine trisphosphate (GTP)-binding proteins called G-proteins.

Signal Transduction Mechanisms.

(i) G-Proteins.

G-proteins are a family of heterotrimers composed of alpha, beta and gamma subunits (Northup et al., 1980; Codina et al., 1984) named in order of their decreasing mass. The alpha subunits are unique for each G-protein with at least seven distinct cDNAs cloned. The beta and gamma subunits are far less diverse, and exist as two subtypes of complexes (beta 36-gamma and beta 35-gamma, with the numbers referring to the mass of the beta subunits in KDa) (Brown and Birnbaumer, 1988). Unlike the alpha subunits, the beta-gamma subunits are functionally interchangeable (Brown and Birnbaumer, 1988).

In the resting state, the alpha subunit is associated with the beta and gamma subunits forming the heterotrimer of the G-protein. The alpha subunit in the resting state has a GDP molecule bound to it. The activation of G-proteins entails conformational changes that result in the dissociation of GDP from the alpha subunit, and the binding of GTP in an exchange reaction (Gilman, 1984; 1987). This is followed by a subunit dissociation reaction that yields a specific activated alpha-GTP complex plus the beta-gamma dimer (Northup et al., 1983; Gillman, 1984; 1987), i.e.

$$\text{alpha-GDP-beta-gamma} + \text{GTP} \xrightarrow{\text{H-R}} \text{alpha-GTP-beta-gamma} + \text{GDP} \Rightarrow \text{alpha-GTP} + \text{beta-gamma} + \text{GDP}.$$

Where H-R represents the formation of hormone-receptor complexes.

The active alpha-GTP complexes are deactivated by hydrolysis of GTP to GDP (Cassel and Selinger, 1981) as a result of intrinsic GTPase activity found in all alpha-subunits (Hildebrandt et al., 1985; Sunyer et al., 1984). This results in the generation of inactive alpha-GDP complexes which reassociate with the beta-gamma dimers to restore the heterotrimeric structure of the G-proteins, and make them ready to reinitiate the cycle under the influence of ligand-occupied receptors.

The activated G-proteins regulate a number of cellular processes and enzymes (Johnson and Dhanasekaran, 1989). These include the disscciation of hormones from their receptors (Johnson and Dhanasekaran, 1989), plus the activation of phospholipase C (Perrin et al., 1989), phospholipase A (Okajima and Ui, 1984), adenylate cyclase (Peake and Smoake, 1985) and calcium channels (Brown and Birnbaumer, 1988).

An association of the GnRH pituitary receptor with GTP-binding proteins was suggested by the observation that the nonhydrolysable guanylyl nucleotides stimulated the accumulation of inositol phosphates and LH secretion (Perrin et al., 1989). These observations, in turn, suggested that the GnRH receptor was coupled to a G-protein, which activated phospholipase C, initiating the hydrolysis of phospholipids resulting in the generation of inositol trisphosphate (IP_3), and the mobilization of calcium (Perrin et al., 1989). Additionally, the binding of GnRH agonists to bovine pituitary receptors was specifically inhibited by guanylyl nucleotides. The binding was inhibited 50% by micromolar concentrations of 5'-guanylyl imidodiphosphate (Gpp(NH)p) and GTP (Perrin et al., 1989). Gpp(NH)p enhanced the rate of dissociation of the ligand

from the receptor and resulted in a 6-fold decrease in the affinity and a 2-fold increase in total number of binding sites (Perrin et al.,1989).

The binding of GnRH to its receptor also increases cAMP production (Bourne and Baldwin, 1987a&b). A potential involvement of G-proteins in this process was suggested by the demonstration that flufenamate inhibited GnRH-stimulated increases in cAMP production (Bourne and Baldwin, 1987a&b; Bourne et al., 1990), an effect which appears to be exerted at the level of the G-protein (Bourne et al., 1990).

Thus, the formation of GnRH-receptor complexes presumably results in the activation of G-protein(s) which may be involved in the generation of some of the intracellular mediators of GnRH actions. The implicated intracellular mediators of GnRH actions include: calcium, IP_3 (and possibly other inositol phosphates), DAG, cAMP, cGMP and the metabolites of AA [such as 5-hydroxy eicosatetraenoic acid (5-HETE) and leukotriene C_4]. Since the focus of this study is on the calcium messenger system, the other postulated intracellular mediators will just be mentioned briefly.

(ii) Cyclic Adenosine Monophosphate (cAMP).

Considerable controversy pervades the literature concerning the role of cAMP as a mediator of the actions of GnRH. Several studies have indicated that adenylate cyclase activity and/or cAMP production was increased by GnRH (Borgeat et al., 1972; Labrie et al., 1973; Makino, 1973), whereas others have failed to demonstrate any effect of GnRH on cAMP production (Berault et al., 1980; Clayton et al., 1970; Naor et al., 1975; Conn et al., 1979). Additionally, while many studies which

utilized cAMP derivatives and agents which alter intracellular cAMP levels support the concept that cAMP mediates LH release (Labrie et al., 1973; Makino, 1973; Tang and Spies, 1976), many other studies have not supported this concept (Sundberg et al., 1976; Stern and Conn, 1981; Ratner et al., 1976). However, recent studies from this laboratory have begun to shed some light on the discordant results reported for cAMP (Bourne and Baldwin, 1987 a&b; Bourne, 1988).

Results of these more recent studies, indicated that GnRH increased cAMP production in pituitaries obtained from both male and female rats, although the increases in females were only detected in the presence of dopamine (which reduces background levels of cAMP by inhibiting cAMP production in activated mammotrophs) (cf. Adams et al., 1979; Bourne and Baldwin, 1987a), and required higher concentrations of GnRH than that observed for pituitaries of male rats (Bourne and Baldwin, 1987a&b). Apparently, dopamine was not required for the demonstration of a GnRH effect in pituitaries of males because there are fewer mammotrophs in male anterior pituitary glands (Bourne and Baldwin, 1987a&b). Furthermore, Bourne (1988) reported that extracellular calcium was required for a GnRH-stimulated increased production of cAMP in gonadotrophs of male but not female rats.

With regard to the role of cAMP as a mediator of the secretion of LH and FSH, the nucleotide does not appear to be involved in the acute release (phase I) of the gonadotropins from female pituitaries, but has a pivotal but indirect role in the second phase release of the hormones (Bourne and Baldwin, 1987a), an effect which involves de novo protein synthesis (Bourne and Baldwin, 1987a). In contrast, cAMP is an indirect

mediator of all phases of gonadotropin secretion from male pituitaries (Bourne and Baldwin, 1987b). As was the case with females, the effects of the cyclic nucleotide were associated with de novo protein synthesis (Bourne and Baldwin, 1987b).

The results obtained from the studies mentioned above (Bourne and Baldwin, 1987a&b; Bourne 1988) have made it possible to reconcile most of the numerous discrepancies in the literature concerning the role of cAMP. For the most part, the discrepancies can be attributed to sex differences in the activation of adenylate cyclase, as well as the roles of cAMP in the different phases of secretion (Bourne and Baldwin, 1987b).

(iii) Cyclic Guanosine Monophosphate (cGMP).

GnRH stimulates cGMP production in hemipituitaries, dispersed pituitary cells and gonadotroph-enriched cell fractions (Snyder et al., 1980; Naor and Catt, 1980). The concentration of cGMP in these preparations correlated well with receptor occupancy by the releasing hormone (Naor and Catt, 1980), and were markedly dependent upon extracellular calcium concentrations (Catt et al., 1983).

Although GnRH consistently increases cGMP production in the anterior pituitary, the secretion of LH was dissociated from increased intracellular concentrations of cGMP. Blockage of cGMP synthesis by mycophenolic acid did not impair the LH response to GnRH (Naor and Catt, 1980). Furthermore, elevation of cellular cGMP levels by nitroprusside or administration of 8-bromo-cGMP did not stimulate LH release (Naor and Catt, 1980). This absence of a cGMP effect on LH secretion, resulted in the suggestion that cGMP may act as a negative, rather than a positive

messenger, providing immediate negative feedback control that prevents an over response (Naor and Catt, 1980; Haslam et al., 1980). In partial support of this view is the observation that sodium nitroprusside which increases cGMP concentrations, is a powerful inhibitor of platelet release of serotonin (Haslam et al., 1980).

In summary, cGMP is a good indicator of gonadotroph activation by GnRH, but does not appear to directly participate in the mechanisms responsible for GnRH-stimulated LH secretion. However, there is a possibility that this cyclic nucleotide might have a negative feedback role.

(iv) Arachidonic Acid and its Metabolites.

Arachidonic acid (AA) and/or its metabolites appear to mediate GnRH-stimulated LH release (Bell et al., 1979 ; Naor et al., 1985a; Catt et al., 1983; 1985). This was illustrated by the demonstration that AA release from prelabelled phospholipids is enhanced by GnRH in cultured pituitary cells (Naor and Catt, 1981), and the administration of the compound caused a dose-dependent increase in LH release even when administered in calcium-free medium (Naor and Catt, 1981). In addition, the lipooxygenase metabolites of AA, 5-hydroxy eicosatetraenoic acid (5-HETE), 15-HETE and leukotriene C_4 (LC_4) have been reported to stimulate gonadotropin secretion from perifused anterior pituitary cells (Catt et al., 1985). In contrast, the addition of other free fatty acids including palmitate and stearate and a non-metabolizable analog of AA (5,8,11,14-eicosatetraenoic acid) were all ineffective in evoking LH release (Naor and Catt, 1981).

Inhibitors of phospholipase A_2 (chloroquine and quinacrine) which decrease the production of AA, reduced GnRH-stimulated LH secretion (Naor and Catt, 1981; Catt et al., 1983; Naor et al., 1985a). Likewise, inhibitors of lipooxygenase pathway which decrease the production of 5-HETE, 15-HETE and LC_4 reduced the LH responses (Naor and Catt, 1981; Catt et al., 1983; Naor et al., 1985a). Taken together, these results suggest that lipooxygenase metabolites of AA might be involved in mediating gonadotropin secretion.

Prostaglandins (PG) are lipid-soluble compounds produced from AA via the cyclooxygenase pathway. However, prostaglandins do not appear to be involved in the mechanism of action of GnRH, since they do not stimulate gonadotropin release (Naor et al., 1983), nor do they appear to be increased during GnRH action. In addition, the cyclooxygenase inhibitor (indomethacin at low concentrations, <50 ug/mL) did not interfere with GnRH-induced gonadotropin release (Naor and Catt, 1981).

Taken together, these observations suggest that only the products of the lipooxygenase pathway of arachidonic acid metabolism and not the products of the cyclooxygenase pathway might actually function as mediators of GnRH action (Naor and Catt 1981; Catt et al., 1983).

(v) Calcium.

In contrast to a number of other intracellular mediators, calcium is commonly accepted as a second messenger for GnRH-stimulated gonadotropin release (Bates and Conn, 1984 ; Conn et al., 1987a). Messenger calcium ions can actually be derived from extracellular or intracellular sources. Since the cytosolic calcium concentration is very low [0.1 uM, (Berridge

,1984)] compared to the extracellular fluid (1000 uM), there is a large concentration gradient for calcium diffusion into the cells upon the arrival of appropriate stimuli. In addition, calcium can also be mobilized from intracellular calcium stores like the endoplasmic reticulum (Rasmussen and Barrett, 1984; Berridge and Irvine, 1984).

The availabilty of enriched gonadotroph cell fractions (cf. Hyde et al., 1982), and the highly fluorescent calcium indicators (Quin II and Fura II) have facilitated the demonstration of changes in the intracellular calcium concentrations in gonadotrophs in response to stimulation. Initially, Clapper and Conn (1985) reported that GnRH stimulates an increase of intracellular calcium in suspensions of enriched gonadotroph pituitary cells. Subsequent studies indicated that the responses actually consist of rapid oscillations in cytosolic calcium concentrations (Naor et al., 1988). It now appears that GnRH specifically stimulates a biphasic response with respect to cytosolic calcium concentrations (Naor et al., 1988; Shangold et al., 1988). The response is characterized by an initial rapid rise of cytosolic calcium which lasts about 8 s before calcium concentrations return to basal levels. This is subsequently followed by a second phase which forms a prolonged plateau (15 min) of elevated cytosolic calcium. Furthermore, the initial response appears to be due to mobilization of intracellular calcium, while the second component consists of extracellular calcium entering via nitrendipene-sensitive voltage-gated calcium channels (Naor et al., 1988; Chang et al., 1986; Shangold et al., 1988).

Evidence that the initial increase of cytosolic calcium is due to calcium mobilization from intracellular stores was obtained from the observations that the peak cytosolic calcium concentration was reached within 8 s of GnRH administration in the presence or absence of extracellular calcium (Naor et al., 1986). Since GnRH stimulates a rapid and specific phospholipase-C hydrolysis of phosphatidyl inositol bisphosphate into IP_3 and DAG (Huckle and Conn, 1987; Morgan et al., 1987), and IP_3 is known to mobilize calcium from the endoplasmic reticulum, it was suggested that IP_3 releases calcium from this organelle, initiating the GnRH-induced initial phase of increased cytosolic calcium concentration increases (Guillemette et al., 1987). In this regard, it is important to note that the activation of phospholipase C is not dependent on extracellular calcium (Huckle and Conn, 1987; Andrews and Conn, 1986; Naor et al., 1986), and that the time course of IP_3 formation by GnRH (5-10 s) is in good agreement with the observed peak elevation in tosolic calcium concentrations (Naor et al., 1986). With respect to the source of calcium for the second phase, the removal of extracellular calcium or addition of a dihydropyridine calcium channel blocker (nitrendipine) completely inhibited the secondary rise in cytosolic calcium concentrations, but had no effect on the initial increase (Shangold et al., 1988). Interestingly, this secondary rise which appears to involve an influx of extracellular calcium, was enhanced by phorbol esters in a nitrendipine-sensitive fashion (Shangold et al., 1988). Thus, it appears that the response to GnRH consists of an initial increase of cytosolic calcium which is mobilized from intracellular stores, followed by a second phase that is due to an influx

of extracellular calcium through calcium channels that may be activated and/or modulated by PKC (Shangold et al., 1988).

The coupling of an increase in cytosolic calcium concentrations to gonadotropin secretion was demonstrated by studies in which any agent which elevated intracellular calcium also provoked gonadotropin release in a time- and dose-dependent manner (Conn et al., 1979b; 1980). These include: i) calcium ionophores e.g. A-23187 and ionomycin (Hopkins and Walker, 1978) allow calcium to enter the cytosol by moving down transmembrane concentration gradients; ii) increased extracellular potassium concentrations (60 mM) depolarize the cell membranes and allow calcium to move into the cells (Hopkins and Walker, 1978; Kraicer, 1975); iii) calcium-loaded, lipid-micelles elevate intracellular calcium by releasing calcium in the cytosol (Conn et al., 1979b); vi) ouabain inhibits the sodium/potassium ATPase and increases cytosolic sodium concentrations (Conn et al., 1981d); the increases in cytosolic sodium activate a calcium/sodium antiporter resulting in an increase in intracellular calcium concentrations, and v) the vincal alkaloid veratridine also provokes LH release by elevation of cytosolic calcium concentrations (Conn and Rogers, 1980).

Therefore, it appears that GnRH elevates cytosolic calcium concentrations by causing calcium release from intracellular stores, and activating calcium channels in plasma membranes, thereby allowing calcium to diffuse into the gonadotrophs. The increased cytosolic calcium concentration (the calcium signal) is then coupled to the secretion of the gonadotropins. The coupling of the calcium signal is actually relayed by two distinct systems, a calcium-calmodulin system, and calcium-activated,

phospholipid-dependent protein kinase (protein kinase C).

The Calcium-Calmodulin System.

Calmodulin is a ubiquitous cytoplasmic protein that binds calcium reversibly with a high affinity and specificity (Rasmussen and Barrett, 1984; Means and Dedman, 1980). As a result of binding calcium, the molecule can then interact with a number of enzymes, induce conformational changes in the enzymes and hence regulate their activity. Calmodulin exists as a monomer containing 148 amino acids, and has a mass (in the rat) of 17000 Da (Means and Dedman, 1980). The calmodulin molecule lacks three amino acids (cysteine, hydroxyproline and tryptophan), and, as a result, the molecule has a high degree of flexibility which facilitates its interactions with a number of other proteins (Means and Dedman, 1980). Additionally, it is an acidic protein with a large number of amino acids which have acidic side chains (e.g. glutamic and aspartic acids). The carboxylic groups of the side chains of these amino acids cluster together to form the calcium binding sites. Calmodulin contains four calcium binding sites. The affinities of these binding sites vary considerably. Binding of calcium to any of the four binding sites results in conformational changes in the molecule which induce changes in the binding affinity of the other calcium binding sites (i.e. the molecule exhibits homologous cooperativity) as well as in the ability of the calcium-calmodulin complex to bind to other proteins (Means and Dedman, 1980; Rasmussen and Barrett, 1984).

Calmodulin regulates the activities of many enzymes (Cheung, 1980).

Such enzymes include the calcium ATPases, adenylate cyclase, cyclic nucleotide phosphodiesterases, phospholipase A_2 and several protein kinases (Rasmussen and Barrett, 1984; Means and Dedman, 1980). The regulation of the activities of enzymes by calmodulin involves the binding of calcium to calmodulin following an increase in cytosolic calcium concentrations. The ensuing conformational change results in an increased affinity for calcium. As a result of the formation of the calcium-calmodulin complex, the calmodulin molecule assumes a more helical conformation which increases the affinity for other proteins and enzymes, thereby facilitating their interactions. The binding of calmodulin to the enzyme induces a conformational change in the enzyme, and hence an alteration in its activity (Rasmussen and Barrett, 1984). It is generally accepted that at least three of the four calcium binding sites on calmodulin must be occupied to activate an enzyme, however, the exact sequence with respect to the number of calcium ions bound prior to interactions between calcium-calmodulin complexes and the enzymes varies, and depends on the enzyme in question (Rasmussen and Barrett, 1984).

Administration of GnRH to ovariectomized (OVX) rats caused an increase in calmodulin in plasma membrane fractions, and a concomitant reduction in cytosolic fractions (Conn et al., 1981c). These effects were both dose- and time-dependent [maximal effect observed 15 min after GnRH administration (Conn et al., 1981c)], and are consistent with translocation of calmodulin from the cytoplasm to the plasma membrane. Other studies (Jennes et al., 1985), also showed an association of calmodulin with GnRH-receptor patches.

Calmodulin inhibitors of several chemical classes (penfluoridol,

pimozide, chlordiazepoxide, chlorpromazine and naphthalene sulphonamide (W-compounds) have all been shown to inhibit GnRH-stimulated LH secretion (Conn et al., 1981d; 1984a). The potency of these compounds at inhibiting LH release correlated well with their potency at inhibiting calcium-calmodulin activation of cyclic nucleotide phosphodiesterase (Conn et al., 1981d). Taken together, these results suggest that calmodulin plays an important role in GnRH-stimulated gonadotropin secretion.

Protein Kinase C .

The second branch of the calcium messenger system is mediated by calcium-activated phospholipid-dependent protein kinases known as protein kinase C (PKC). PKC is actually a group of enzymes that catalyse the covalent binding of a phosphate group derived from ATP to a protein substrate. In vitro, PKC has a broad substrate specificity, phosphorylating seryl and threonyl but not tyrosyl residues of many endogenous proteins in most tissues (Nishizuka, 1986; Nishizuka et al., 1984). The primary sequence in the vicinity of the phosphorylation site, as well as the topographical localization of the enzymes are important in determining substrate recognition in the different protein kinases (Nishizuka, 1988). Phosphorylation of these protein substrates results in changes in their conformation and enzymatic activity.

PKC is representative of a family of related gene products (proteins) (Nishizuka, 1988; Parker et al., 1989). Seven subspecies of PKC have been identified. Four subspecies (alpha, beta I, beta II, and gamma) emerged from the initial screening of a variety of DNA libraries. More recently, at least three other subspecies (delta, eta and zeta) have been

isolated from a rat brain library by using a mixture of alpha, beta II and gamma DNA as probes under low stringency conditions (Nishizuka, 1988). To date the latter three subspecies have not actually been purified from cells (Barker et al., 1989). All of the enzymes consist of a single polypeptide chain (molecular weight 77000 Da) that has two functionally different domains which can be separated by calcium-dependent thiol proteases (Kikkawa et al., 1982; Kishimoto et al., 1983). One is a hydrophobic regulatory domain that is thought to interact with the plasma membranes, while the other is a hydrophilic domain that contains the catalytically active center (Takai et al., 1979; Nishizuka, 1984). The separated catalytic domain which is fully active in the absence of calcium, phospholipids or DAG is rapidly removed from the cell (Nishizuka, 1984). The PKC molecules demonstrate four conserved (C) regions and five variable (V) regions. The conserved region C_1 contains the DAG binding site and forms a part of the regulatory domain of PKC. The carboxy terminal half of each enzyme containing the conserved regions C_3 and C_4 seems to be the catalytic domain. The conserved region C_3 is the sequence which has an ATP-binding site. Different protein kinase C subspecies contain different variable (V) regions (Nishizuka, 1988).

The C_2 region is completely missing in all the three predicted polypeptides (delta, eta and zeta), although a cysteine-rich sequence is retained (Nishizuka, 1988). Because the last three peptides were not actually isolated from cells but are only predicted by cDNA studies, the discussion on activation will be limited to the alpha, Beta I, Beta II and gamma subspecies.

Protein kinase C alpha, beta and gamma subspecies are all calcium/phospholipid/DAG-dependent protein kinases (Knopf et al., 1986; Kikkawa et al., 1987). There are functional differences between the members of PKC family. These functional variations are concerned both with the potency with which the physiological activators of PKC will act, and with the potential targets for activated kinase activities. The differences between the alpha, beta and gamma subspecies are summarized in Table 1.

Activation of Protein Kinase C.

The formation of GnRH-receptor complexes in gonadotrophs results in the activation of G-proteins which consequently activate phospholipase C and result in the hydrolysis of phosphatidyl inositol 4,5 bisphosphate into IP_3 and DAG (Huckle and Conn, 1987). Under resting conditions, diacylglycerol is normally almost absent from the membranes, but is transiently produced from inositol phospholipids in response to extracellular signals (Nishizuka, 1984; Bell, 1986). It is rapidly removed within a few seconds or at most several minutes after its formation (Kishimoto et al., 1980), presumably due to its conversion back to inositol phospholipids by way of phosphatidic acid (PI turnover) and/or removal by further degradation to AA (Nishizuka, 1984). However, the transient appearance of DAG in membranes is usually associated with translocation of PKC from the cytosol to the plasma membrane and activation of PKC. DAG binds to protein kinase C presumably on the regulatory domain in the C_1 region (Parker et al., 1989), following its translocation to the membrane. In the presence of phosphatidyl serine and other membrane phospholipids, DAG dramatically increases the affinity

Table 1.

Functional differences between the different protein kinase C subspecies.

Character	Alpha	Beta I/II*	Gamma	Refrences
Dependence on DAG or Phorbol esters for activation	Yes	Yes	Yes	Huang et al.,1988
Sensitivity to PMA & DAG**	5X less affinity than BI/BII	more sensitive	Least sensitive	Parker et al., 1989
Sensitivity to PKC activators when PS is replaced by PM or PI			more sensitive	Asaoka et al. 1988
Calcium Dependence ***		significantly reduced relative to alpha or gamma		Kosaka et al.,1988

* There is much more B II than BI in endocrine tissues including the pituitary gland.

** The variation in response of PKC isotypes to DAG and phorbol esters under different conditions implies that there are real differences between the isotypes in binding of these effectors.

*** The differences in calcium dependence between PKC isotypes may reflect substrate directed specificities (Parker et al., 1989). Some substrates can be phosphorylated in a calcium-independent manner (Nunn and Watson, 1987).

of PKC to calcium resulting in the binding of calcium to the enzyme. The calcium-PKC complex represents the activated enzyme which phosphorylates substrate proteins, thereby altering cellular functions (Nishizuka, 1984; Rasmussen and Barrett, 1984). It is noteworthy that DAG increases PKC sensitivity to calcium such that the enzyme can be activated in the absence of detectable cellular calcium mobilization (Conn et al., 1985; Bourne et al., 1989; Nishizuka, 1984; Rasmussen and Barrett, 1984; Yamanishi et al., 1983), or phosphatidyl inositol turnover (Castanga et al., 1982).

Protein kinase C can also be activated by limited proteolysis which cleaves the PKC molecule into its regulatory and catalytic components (Kishimoto et al., 1983). The smaller catalytic component is enzymatically active totally independent of calcium, phospholipid or DAG and is rapidly removed from the cell (Nishizuka, 1986). Membrane-bound PKC is more susceptible to this kind of proteolysis (Kishimoto et al., 1983), suggesting that the physiological significance may be related to the down-regulation of PKC (Drummond, 1985).

Castanga and collegues (1982) discovered that the phorbol esters [e.g. phorbol, 12-myristate 13, acetate (PMA)] can substitute for DAG as an activator of PKC (Castanga et al., 1982; Nishizuka, 1983; Yamanishi et al., 1983). Like DAG, PMA dramatically increases the affinity of the enzyme for calcium (in the presence of phosphatidylserine) resulting in its full activation without detectable cellular mobilization of calcium (Yamanishi et al., 1983). The only major difference in the activation of PKC by DAG and PMA is the fact that PMA is much more potent than DAG since it is not rapidly metabolized like the DAGs (Michell, 1983).

Role of Protein Kinase C in Gonadotropin Secretion.

Although, it was initially suggested that PKC is not involved in the LH response to GnRH (McArdle et al., 1987), later reports by Naor et al., 1987, Stojilkovic et al. (1988), Mc Ardle et al. (1988), Bourne et al. (1989) and Das et al. (1989), support the concept for PKC involvement as a mediator of GnRH-induced gonadotropin release. In brief, the evidence which suggests an involvement of PKC in GnRH-stimulated gonadotropin release includes:

1. PKC is present in the anterior pituitary (Turgeon et al., 1984). GnRH or activators of PKC like DAG or phorbol esters, cause the redistribution of the enzyme from the cytosol to the plasma membranes of pituitary gonadotrophs (Hirota et al., 1985; Naor et al., 1985b; Stojilkovic et al., 1988).

2. Redistribution of PKC by GnRH or PMA is associated with increased synthesis and release of gonadotropins (Smith and Vale, 1980; Turgeon and Waring, 1986; Negro-Vilar and Lapetina, 1985; Stojilkovic et al., 1988). The newly synthesized LH and FSH contributed significantly to the total amount of gonadotropins available for release (Stojilkovic et al., 1988). The ratio of the newly synthesized to the released LH was constant (1:2) during stimulation by increasing concentrations of PMA (Stojilkovic et al., 1988).

Taken together, the above-mentioned observations indirectly suggest a possible role of PKC as a mediator of gonadotropin secretion. It should be emphasized however, that while there is now common agreement that PKC is a mediator of some of GnRH actions, there is still some disagreement as to whether the enzyme mediates gonadotropin secretion (cf. Conn, 1989).

Based on the discussion above, it should be apparent that the calcium signal in gonadotrophs is relayed by two distinct systems, a calcium-calmodulin system which is activated by transient increases in cytosolic calcium, and PKC which is activated by changes in the cellular concentration of DAG. It is noteworthy that PMA can substitute for DAG in sensitizing PKC to calcium, such that the enzyme is activated without causing a rise in cytosolic calcium (Rasmussen and Barrett, 1984; Nishizuka, 1984). Equally interesting is the fact that ionomycin will increase cytosolic calcium and activate calmodulin without activating PKC (Rasmussen and Barrett, 1984). Therefore, the experimental tools are available to selectively activate either one or both arms of the calcium messenger system. Consequently, the initial objective of this study was to use PMA and ionomycin to delineate the contribution of the calcium messenger system towards the secretion of LH and FSH from the anterior pituitaries of male rats. However, as a result of the initial findings, the objective was expanded to investigate a modulatory role of estradiol on the PMA- and ionomycin-stimulated mechanisms.

Role of Estradiol as a Modulator of Gonadotropin Secretion.

Serum estradiol levels in vivo fluctuate with the estrous cycle of the rats e.g. in estrous rats the estradiol levels are lowest (< 5 pg/mL) and it increases gradually during the estrous cycle to reach a level around 40 pg/mL during the diestrous II (DII) and peaks at 60-75 pg/mL at proestrus. These estradiol levels have been shown to affect gonadotropin secretion (Brown-Grant, 1977; Knobil, 1980). In males the estradiol concentrations are low and are almost similar to the estrous females (< 5

pg/mL).

As stated above, estradiol exerts a biphasic effect (inhibition followed by stimulation) on LH and FSH secretion during the reproductive cycles of mammals. The effects of estradiol are exerted at both the level of the hypothalamus and the level of the anterior pituitary gland (Brown-Grant, 1977; Knobil, 1980). For example, in ovariectomized rats, estradiol initially suppresses the pituitary gonadotropin response to GnRH (Schuiling and Gnodde, 1977), but in the continued presence of estradiol, the response increases and finally becomes augmented (Schuiling and Gnodde, 1976).

Estradiol exerts a transient inhibition of GnRH-stimulated LH secretion by a direct effect on the rat pituitary. These inhibitory effects of estradiol were demonstrated in vitro in static cultures of rat pituitary cells, provided that the period of exposure to estradiol was brief (Frawley an Neill, 1984; Fink and Pickering, 1980). The stimulatory effects of estradiol were also demonstrated in vitro, since the steroid augmented the GnRH-induced LH release in static cultures of rat pituitary cells (Drouin et al., 1976; Hsueh et al., 1979; Tang, 1980; Kamel and Krey, 1982).

It is commonly believed that estradiol enhances gonadotropin secretion by affecting signal transduction mechanisms through processes which involve protein synthesis (de Koning et al., 1976a; Debeljuk et al., 1978). The estradiol-induced effects include an influence on the degree of sialic acid incorporation into gonadotropin molecules, thereby influencing the type of gonadotropin that is being synthesized and secreted (Ulloa-Aguirre et al., 1984; Peckham and Knobil 1976). In

addition to affecting gonadotropin biosynthesis, estradiol was also reported to stimulate the synthesis of cAMP-binding proteins (Szabo and Endoroczi, 1981), increasing both cAMP binding and pituitary cAMP-dependent protein kinase activity (Tang and Tang, 1979).

The observation that estradiol can affect signal transduction mechanisms in gonadotrophs, coupled with my initial findings related to the role of calcium (see below), resulted in an expansion of my research objectives to include investigations designed to determine whether estradiol could affect the calcium messenger system (calcium-calmodulin and PKC) by protein synthesis-dependent mechanisms in pituitaries of male rats.

C. LIST OF SPECIFIC OBJECTIVES

1. To investigate the estradiol-dependency of the PMA-stimulated gonadotropin secretion, and to test the dependency of this phenomenon on de novo protein synthesis.

2. To investigate whether estradiol can induce an extracellular calcium-independent component of gonadotropin secretion, and test whether this secretion is dependent on de novo protein synthesis.

3. To characterize the ionomycin-induced gonadotropin secretion from pituitaries of male rats, and to investigate potential interactions of the PMA- and ionomycin-activated mechanisms in these gonadotrophs, while concomitantly determining whether they are dependent on de novo protein synthesis.

CHAPTER II

MATERIALS AND METHODS

A. Materials.

The materials used in this study were as follows:

Biogel P-100 (100-200 mesh wet) (Bio-Rad Laboratories, Richmond, CA); ionomycin (calcium salt) (Calbiochem Corporation, La Jolla, CA); Eagle (Modified) minimum essential amino acids without glutamine, Eagle (Modified) minimum essential vitamins, fetal bovine serum and Ham's F10 medium with glutamine (Flow Laboratories, McLean, VA). Staurosporin was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA).

Chemicals purchased from Sigma Chemical Co. (St. Louis, MO) were: bovine serum albumin (BSA) (RIA grade; fraction V), cycloheximide, deoxyribonuclease (DNase) (Bovine pancreatic type III, lyophilized), ethylene glycol bis (β aminoethyl ether) N,N,N',N' tetraacetic acid (EGTA), estradiol-benzoate, sodium pyruvate, sodium fumarate, sodium glutamate, glucose, phorbol 12-myristate, 13-acetate (PMA), soyabean trypsin inhibitor, streptomycin sulfate-penicillin G as potassium salt, prepared in 0.9 g % NaCl, trypsin (Bovine pancreatic type III), pimozide and W7.

Inorganic salts (sodium chloride, potassium chloride, calcium chloride, magnesium sulphate and sodium monobasic phosphate) and dimethyl sulphoxide (DMSO) were bought from Fisher Scientific Corporation (Pittsburgh, PA).

The ^{125}I was purchased from Radiopharmacy, University of Alberta, (Edmonton, AB), while GnRH was obtained from Peninsula Laboratories (Belmont, CA).

PMA was prepared as a concentrated stock solution (1 mM) in an acetone/ethanol mixture following the procedures of Prince et al. (1973), and diluted with perifusion medium on the day of an experiment to give the indicated concentration. Ionomycin was dissolved in 95% ethanol and stored as a stock (1 mM) solution at 5°C. Aliquots of the ionophore were diluted with perifusion medium to give the indicated concentration just before use. Staurosporin was dissolved in dimethyl sulphoxide (DMSO) as a stock solution (1 mg/mL) and stored at 4°C in the dark. The final concentration of staurosporin used in the study was 10 nM.
Pimozide was prepared fresh just before the experiments by dissolving it in DMSO. The final concentration in the perifusion medium was 10 uM. W7 was dissolved in DMSO and the final concentration used in the experiments was 100 uM. The vehicle concentrations (0.1%, v/v) had no measurable effects on LH and FSH secretion. Staurosporin, W7 or pimozide were all added to the medium one hour before infusion of the secretagogues.

B. Methods.

I. Animals and Tissue Preparation.

Adult, Sprague-Dawley rats (Bioscience Animal Services, University of Alberta, Edmonton, AB), 60 to 90-days old were used for all experiments in this study. The rats were housed in a temperature- and light-controlled environment (21 ± 1°C , lights on 06:00 to 18:00 h) with food and water supplied ad libitum.

Depending on the experiment, intact males, castrated males (72 h), diestrous II females showing at least two consecutive four-day estrous cycles (determined by daily vaginal cytology), and ovariectomized (72 h) female rats were used as pituitary donors. Ovariectomies and orchidectomies were performed under light general ether anaesthesia. The orchidectomies were performed by making a small vertical midline scrotal incision through which both testes were removed after the vas and blood vessels were ligated. The scrotal incisions were closed with surgical wound clips. Ovariectomies were accomplished via a transverse incision in the lower back, through which both ovaries and parts of the Fallopian tubes were removed. The muscles were then sutured with surgical silk (size 000), and the skin incisions closed with surgical wound clips.

Estradiol silastic capsules were implanted in some castrated animals for some experiments. The capsules were prepared following the procedures of Legan et al. (1975). Briefly, 1.5 cm of silastic tubing (inner diameter 0.155 cm and outer diameter 0.3105 cm, Dow Corning Corporation, Midland, MI) was used. The tubing was plugged with a 0.5 cm piece of a wooden applicator stick (Canlab, Mississauga, ONT) on one side and then filled with crystalline estradiol-benzoate. After packing the tubing with estradiol, the implants were plugged with another 0.5 cm piece of applicator stick on the other side. Both ends of all implants were sealed with silastic medical adhesive (silicone type, Dow Corning Corporation, Midland, MI) to make the ends of the implants water and air tight. The estradiol silastic implants were incubated overnight in physiological saline at 37°C in a Dubnoff Metabolic Shaker (GCA Corporation, Chicago, IL) before use. The estradiol implants were

inserted (one for each rat) subcutaneously in the back of some gonadectomized rats 24 h before sacrifice. These implants were expected to maintain plasma estradiol at proestrous concentrations [i.e. 60 to 75 pg/mL (Legan et al., 1975 ; Baldwin et al., 1983)]. Provided that the weight of the rats is in the range of 250-300 g, the estradiol implants produced a highly reproducible postimplantation serum estradiol concentration in females similar to that seen in proestrous females (cf. Appendix 1). Slightly higher values were observed in males implanted similarly (Appendix 1).

All animals were killed by decapitation (without anaesthesia) to avoid the hormonal changes that occur due to the stress of anaesthesia. The pituitaries were rapidly excised, the posterior pituitaries dissected out and the anterior pituitaries placed in prewarmed (37 $^{\circ}$C) perifusion medium to minimize temperature-shock (Bourne and Baldwin, 1980).

II. The Perifusion Media.

Kreb's Improved Ringer I Bicarbonate (KIRB) solution [McKenzie and Dawson, 1969; (cf. Appendix 2)] supplemented with minimum essential amino acids and vitamins was used as the perifusion medium. On the day of an experiment, the medium was made up from stock solutions with the exception of the bicarbonate solution which was freshly prepared. The inorganics (NaCl, KCl, $CaCl_2$, $MgSO_4$ and NaH_2PO_4) were added first, followed by the organics (sodium pyruvate, sodium fumarate, sodium glutamate and glucose) and then the amino acids and vitamins. Sodium bicarbonate was added last. In the experiments where cycloheximide was used, it was added before the bicarbonate, and the medium was stirred thoroughly to dissolve

the added cycloheximide. The solution was gassed with 95% O_2 : 5% CO_2 for 10 minutes before adjusting the pH to 7.4 at room temperature by the addition of 10% sodium hydroxide. The calcium-free medium was prepared by simply omitting calcium chloride. As described previously, (Bourne and Baldwin, 1980), 16 uM EGTA was added to the calcium-free medium to chelate any contaminating calcium.

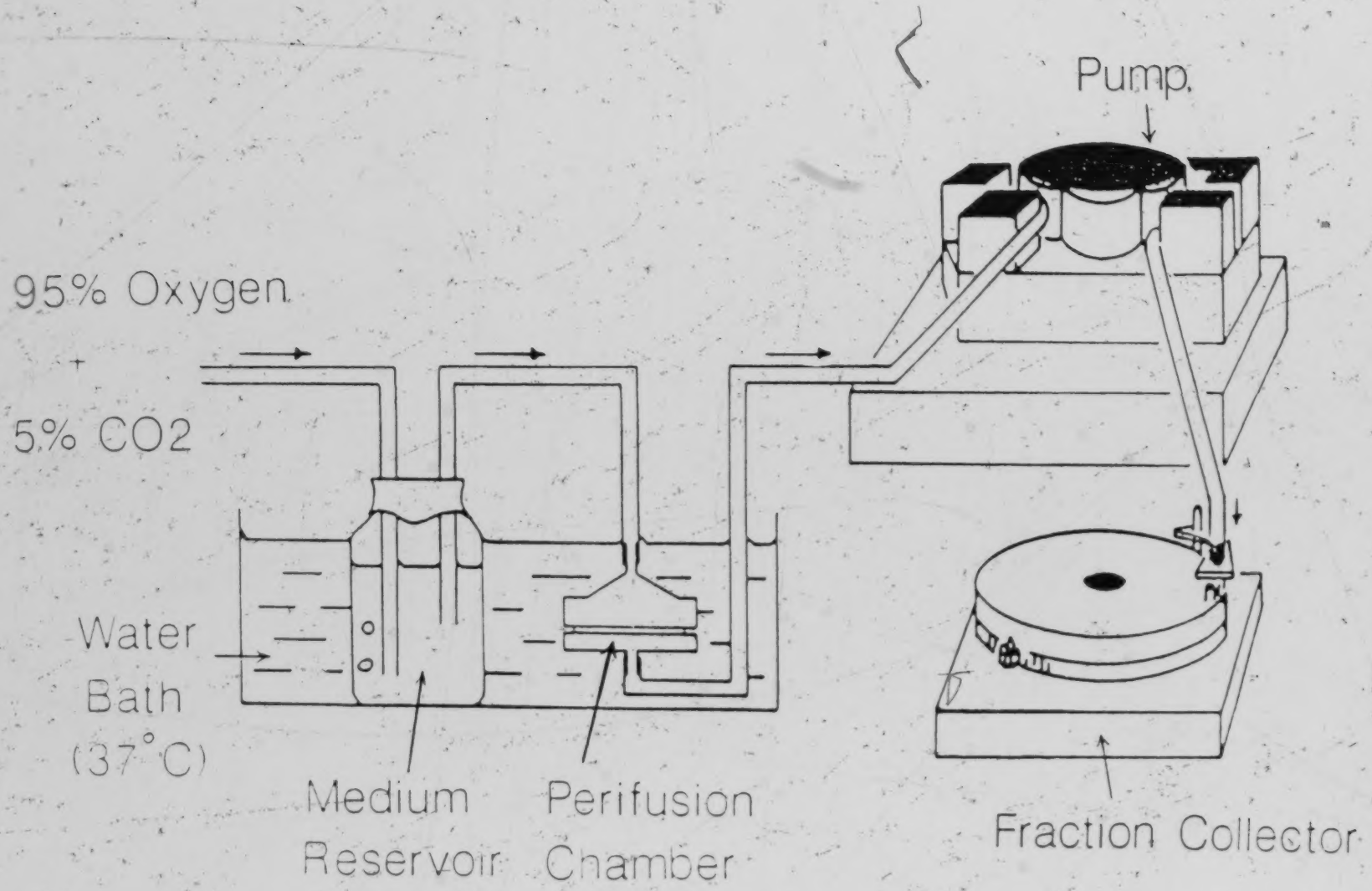
All media were cold-sterilized using disposable Sterivex-GS millipore filters (0.22 um) just before use. During experiments, the medium reservoirs (300 mL each) were placed in a water bath at 37°C, and the medium was continuously gassed with 95% O_2 : 5% CO_2. For those experiments in which light-sensitive chemicals (e.g. ionomycin and PMA) were used, the reservoirs were wrapped in aluminium foil.

III. The Perifusion System.

The anterior pituitary tissue perifusion system described by Bourne and Baldwin (1980) was used in this study. A schematic diagram of the system is shown in Fig. 1. Three to four perifusion chambers (Millipore Swinnex-13 filter holders) were usually run simultaneously for each experiment. Two related experiments were normally undertaken at the same time for a total of six to eight perifusions at any one time. There was no need to run some chambers as controls since each chamber effectively served as its own control (first hour). The perifusion chambers, associated tubings and glassware were all autoclaved before use.

The perifusion chambers were initially charged with medium, immersed in a water bath at 37°C and the system was allowed to equilibrate for one hour (during which time the rat pituitaries were collected). At the end

Fig. 1. Schematic diagram of the anterior pituitary tissue perifusion system. A water bath was used to maintain the perifusion medium and the perifusion chambers at 37 °C. The medium which was continuously gassed with 95% O_2 : 5% CO_2, was pumped (0.25 mL/min) from the reservoir through the perifusion chambers to fraction collectors by a Gilson peristaltic pump. The effluent fractions were collected at room temperature at 10 min intervals for the duration of an experiment (6 h).


Pump
95% Oxygen
+
5% CO2
Water
Bath
(37°C)
Medium
Reservoir
Perifusion
Chamber
Fraction Collector

of this period, air bubbles were expelled from the system by reversing the direction of flow and finger flicking the chambers. The pituitaries were cut into quarters and then loaded into the chambers using a sterile short-tipped Pasteur pipette. Two quartered pituitaries were placed in each perifusion chamber.

A multichannel, peristalitic pump (Minipuls-2, Gilson), maintained the flow of the medium through the system at a constant rate of 0.25 mL/min. Sequential effluent fractions were collected every 10 min (2.5 mL of perifusate) in 2% BSA-coated borosilicate glass tubes using fraction collectors (LKB Redirac Fraction Collectors). The perifusate for the first hour was discarded, since it was previously shown to contain high nonspecific release of hormones from pituitary cells injured as a result of tissue manipulations (Bourne and Baldwin, 1980).

The test compounds were dissolved in the medium and introduced into the perifusion system by stopping the pump and the timer of the fraction collectors and quickly transferring the inlet tubing from the control reservoir to the reservoir with the test compounds. The pump and timer of the fraction collectors were then restarted. The entire operation took less than 15 s.

Since previous experiments (Bourne and Baldwin, 1980) had demonstrated no significant changes in LH immunological activity in aliquots of samples frozen immediately after collection, versus those which were kept at room temperature for the duration of the experiment, all samples were collected at room temperature and stored frozen (-20°C) until assayed for LH and FSH.

IV. Cell Dissociation Procedures.

Dispersed anterior pituitary cells were prepared by enzymatic dissociation of anterior pituitaries from male rats using a modified version of the procedures described by Denef and his collegues (1976). In brief, the rats were decapitated, the anterior pituitaries rapidly excised and collected under sterile conditions in prewarmed (37°C), calcium-magnesium-free Ringer solution containing 3 mg/mL BSA (CMF-BSA). Each pituitary was then cut into nine pieces under a laminar flow hood and the tissue blocks were washed four times with the CMF-BSA solution.

The washed tissue blocks were incubated at room temperature with 5 mL trypsin solution (5 mg/mL trypsin in CMF-BSA) and shaken every 5 min. After 15 min of incubation with trypsin, the tissue blocks were allowed to settle and the supernatent aspirated using sterile Pasteur pipettes. The tissue blocks were then incubated with 5 mL of soyabean trypsin inhibitor (SBTI) solution (1 mg/mL SBTI in CMF-BSA) for 15 min at room temperature with repeated gentle shaking every 5 min. At the end of the SBTI incubation, 1 mL of DNase (10 ug/mL in CMF-BSA) was added and the mixture was incubated for an additional 5 min in a Dubnoff metabolic shaker at 37°C.

After allowing the tissue blocks to settle to the bottom of a conical test tube, the supernatant was aspirated and the tissue was washed three times with CMF-BSA. Mechanical dissociation of the tissue was achieved by gentle trituration using fire-polished siliconized Pasteur pipettes. Whenever the solution became cloudy, the tissue blocks were allowed to settle and the supernatent fluid containing the dispersed cells was

transferred to another container and replaced by 1 mL of CMF-BSA solution. The procedure was repeated until all of the tissue blocks were completely dispersed.

The pooled cell suspension was centrifuged for 10 min at room temperature (300 x g) and the pellet resuspended in a small volume (0.5-1.0 mL) of CMF-BSA medium. After counting the cells with a hemocytometer, they were diluted to the desired concentration using the cell culture medium. The cell yield was on the average 3 million cells per anterior pituitary gland. Cell viability, ascertained by using trypan blue dye exclusion test (Tennant, 1964), was 90-95%.

The resuspended cells were divided into two equal aliquots and diluted with the culture medium to a concentration of 5×10^5 cells/mL. One mL aliquots of the cell suspension were plated into 35 x 10 mm tissue culture dishes (Miles Scientific Corporation, Naperville, IL). Two mL of incubation medium was added to each dish, and the cells were incubated for two days under a humidified 5% CO_2 : 95% air at 37°C using a NAPCO water-jacketed incubator (model 5100).

V. Pituitary Cell Incubations.

Half of the cells was cultured in Ham's F10 medium supplemented with 15% horse serum, 2.5% fetal calf serum, penicillin (50 U/mL) and streptomycin (50 ug/mL), while the other half was cultured in Ham's F10 medium supplemented with 17.5% male rat serum and the antibiotics. The male rat serum was prepared from trunk blood collected during sacrifice of intact male rats. The blood was allowed to clot at 5°C, the serum collected and cold-sterilized using the Sterivex-GS (0.22 um) filters.

After the 2-day incubation period, the medium in each dish was replaced with 2 mL of fresh medium containing either 1 uM PMA or vehicle. Following a 4-hour experimental incubation period, the media were collected, centrifuged and the supernatants collected and stored at -20°C until assayed for LH and FSH.

VI. Radioimmunoassays for LH and FSH.

Samples were assayed for LH and FSH in duplicates using the National Institute for Arthritis, Diabetes, Digestive and Kidney disease (NIADDK) rat radioimmunoassay kits.

(i) Iodination Procedure.

LH and FSH were iodinated with ^{125}I using the chloramine-T method (Hunter and Greenwood, 1962), and the labelled hormones were separated from the unlabelled hormones by gel filtration chromatography. Briefly, columns for the separation of the labelled hormones were prepared using 10-mL disposable borosilicate pipettes, which were packed with G-75 Sephadex beads. The beads were washed, swollen and degassed overnight in 0.05 M sodium phosphate buffer (pH 7.5) prior to use. Just before starting the iodination, 3 mL of 0.05 M EDTA-PBS-1% BSA was run through the column to reduce nonspecific binding. The radioactive iodide (40 mBq ^{125}I/50 uL of 0.5 M sodium phosphate buffer, pH 7.5) was then added to the reaction chamber containing the hormone aliquots. The iodination reaction was initiated by adding freshly prepared chloramine-T (0.2 mg/mL), and the reaction was allowed to proceed for 110 s before termination by addition of 0.05 M EDTA-PBS-1% BSA (pH 7.5). The entire

contents of the reaction vessel were transferred to the Sephadex column and eluted with 0.05 M EDTA-PBS-1% BSA (pH 7.5). Twenty fractions (five drops per fraction) were collected. After counting the fractions, the peak tubes were pooled, aliquoted and stored at -70° C.

(ii) Purification of Iodinated Hormones.

The stored iodinated hormone was purified before use on a Biogel P-100 column. The Biogel P-100 was washed, swollen and degassed overnight in double-distilled water in the cold room (4° C) prior to packing the column (in the cold room). Before using the column initially, it was washed with 0.01 M sodium barbital, 0.15 sodium chloride, 2% BSA, pH 7.6. The thawed hormone aliquots were added to the column and eluted with 0.01 M sodium barbital, 0.15 M sodium chloride, pH 7.5 . Thirty fractions (14-drops each) were collected, counted and the peak tubes were pooled and diluted with phosphate-buffered saline-0.1% gelatin to a concentration of 10,000 cpm/100 uL for use in the assays.

(iii) Radioimmunoassay for LH and FSH .

A 3-day double antibody radioimmmunoassay (RIA) protocol was used. On the first day, the buffer (phosphate-buffered saline-0.1% gelatin, pH 7), the radiolabelled ligand, the antibody and the standards or samples were added to numbered 12 x 75 mm borosilicate glass test tubes. After a 24-hour incubation at room temperature, 100 uL anti-rabbit gamma globulin (ARGG) was added to all tubes which were then incubated for an additional 24 h at 4° C. On the third day of the assay, 1.5 mL cold (4° C) phosphate-buffered saline (PBS, pH 7.0) was added to the tubes which were

centrifuged at 2100 x g for 45 min at 4°C. The supernatants were then aspirated and the gamma radiation was assessed by using a Micromedic 4/200 Gamma Counter.

(iv) Estimation of Sample Concentration.

Sample estimates were calculated using the log-logit transformation and weighted least squares regression analysis as described by Rodbard et al. (1969). Samples of the standard curves are shown in Appendix 3. The values were compared with the NIADDK reference preparations of rat LH-RP-2 and FSH-RP-2. Consequently, the results are 61- and 45-times lower than those obtained in reference to LH-RP-1 and FSH-RP-1, respectively.

The intra- and interassay coefficients of variation were calculated from the estimates of the values of an LH and FSH pool according to the formula:

coefficient of variation = (standard deviation/mean) x 100.

The intraassay and interassay coefficients of variation for LH were 6% and 11%, respectively, while those for FSH were 6% and 10%, respectively.

VII. Radioimmunoassay for Estradiol.

Trunk blood was collected at the time of killing the animals, to produce sera for measurement of estradiol. The blood was allowed to clot at 4°C then centrifuged at 500 x g for 10 min, and the supernatant collected and stored frozen at -20°C until assayed. Estradiol levels were

measured using RIA in the Endocrine Laboratory of the University of Alberta Hospital (Courtesy of Dr. D.M. Fawcett) and in Animal Sciences Department, University of Alberta (Courtesy of Dr. S. Baido). The intra- and interassay coefficients of variation were less than 10%. The results of estradiol assays are shown in Appendix 1.

C. Data Analysis.

Quantitative estimates of the total amounts of hormones secreted in response to continuous infusions of the various secretagogues were calculated by summing the amounts of hormone in the 10 min fractions collected during the period of secretagogue infusion. Before statistical analysis, the values were corrected for basal release unless stated otherwise.

The results were statistically analysed using Analysis of Variance and differences between means determined by the Duncan-Bonner test (Computer program provided by Dr. G.A. Bourne). The level of statistical significance used in this study was $p < 0.05$.

CHAPTER III

RESULTS

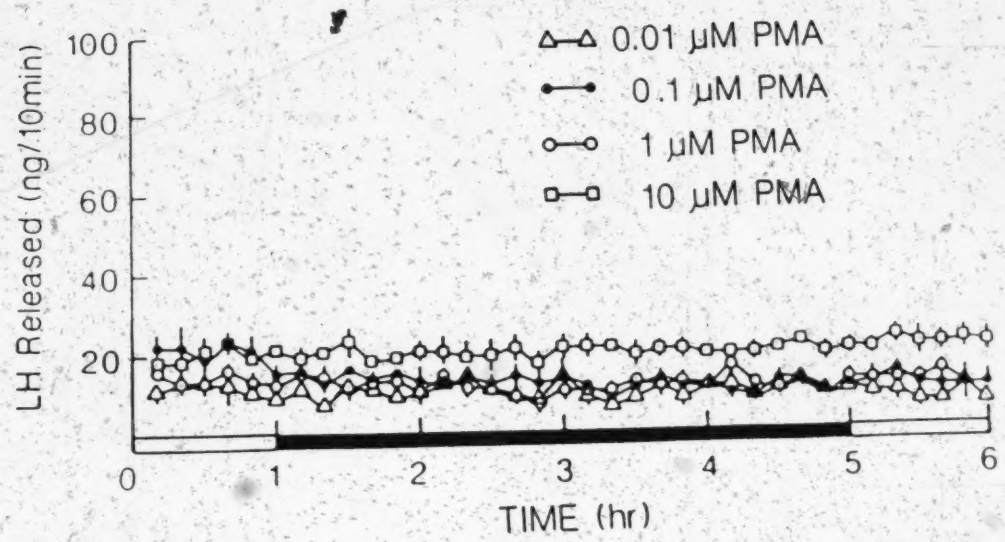
I. Estradiol-Dependency of PMA-Stimulated Gonadotropin Secretion from the Anterior Pituitaries of Male and Female Rats.

The first series of experiments used PMA to investigate the potential role of protein kinase C as a mediator of gonadotropin secretion from anterior pituitaries of male rats. Initial experiments were undertaken to characterize the secretion of LH and FSH from these pituitaries perifused with KIRB medium in response to the phorbol ester, PMA. The results are shown in Fig. 2. Essentially, PMA at concentrations ranging from 0.01 to 10 uM was ineffective in stimulating the secretion of LH and FSH from pituitaries of male rats.

Since previous results had indicated that 1 uM PMA was effective in stimulating gonadotropin secretion from pituitaries of diestrous II females perifused with KIRB medium (Das et al., 1989), pituitaries obtained from intact males, diestrous II females, ovariectomized (OVX) females and ovariectomized + estradiol-treated (OVX + E_2) females were all perifused simultaneously with 1 uM PMA in KIRB medium. As shown in Fig. 3, PMA stimulated the secretion of gonadotropins from pituitaries of both diestrous II and OVX + E_2-treated females, but failed to stimulate gonadotropin secretion from pituitaries of males or OVX females, suggesting that the efficacy of PMA in stimulating gonadotropin secretion depends on estradiol.

Furthermore, the amounts of LH secreted from pituitaries of OVX + E_2 -treated females were greater than the amounts of LH secreted from

Fig. 2. Dose-response of anterior pituitary tissue blocks of male rats to continuous infusion (4 h) of PMA (indicated by the black bar) in KIRB medium. Results are the means ± SEM (n=3). Absence of standard error bars is indicative of the fact that the standard errors are smaller than the size of the symbols. Secretory profiles for LH are shown in the upper panel, whereas those for FSH are depicted in the lower panel. The abbreviations are defined in the text.


0.01 μM PMA
0.1 μM PMA
1 μM PMA
10 μM PMA
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

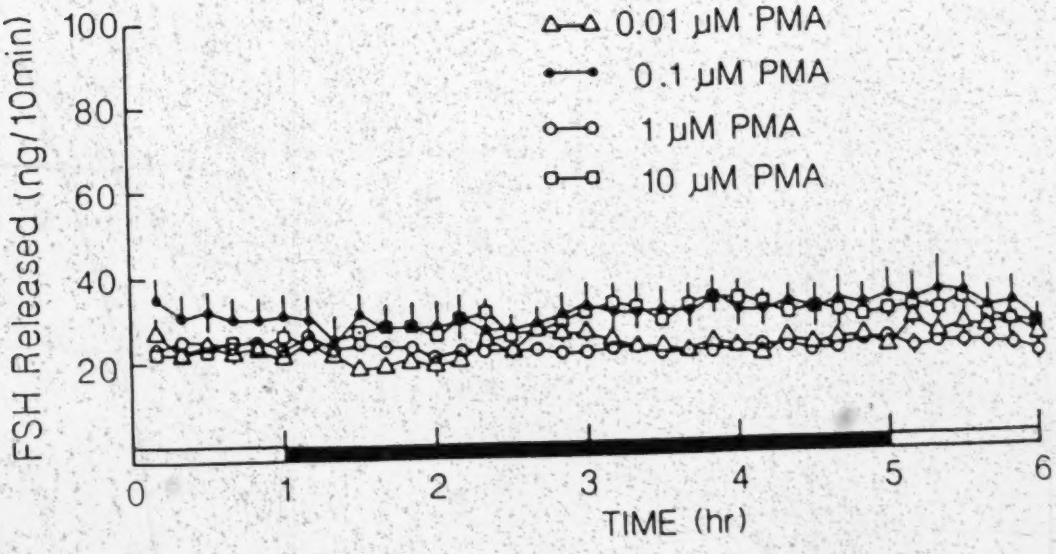
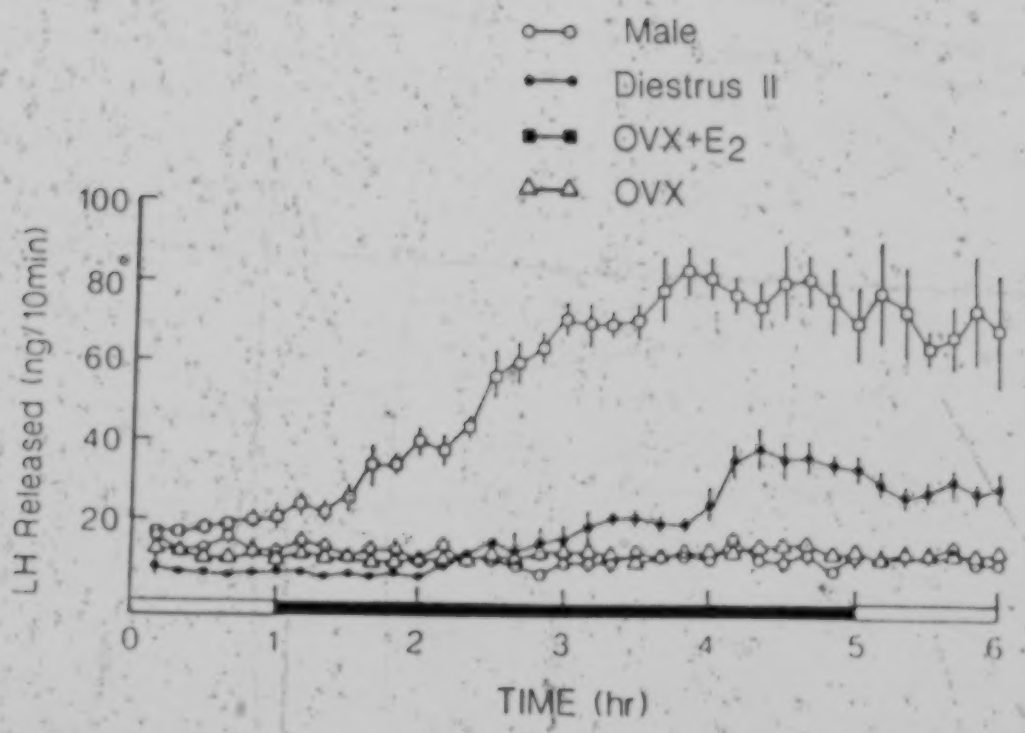
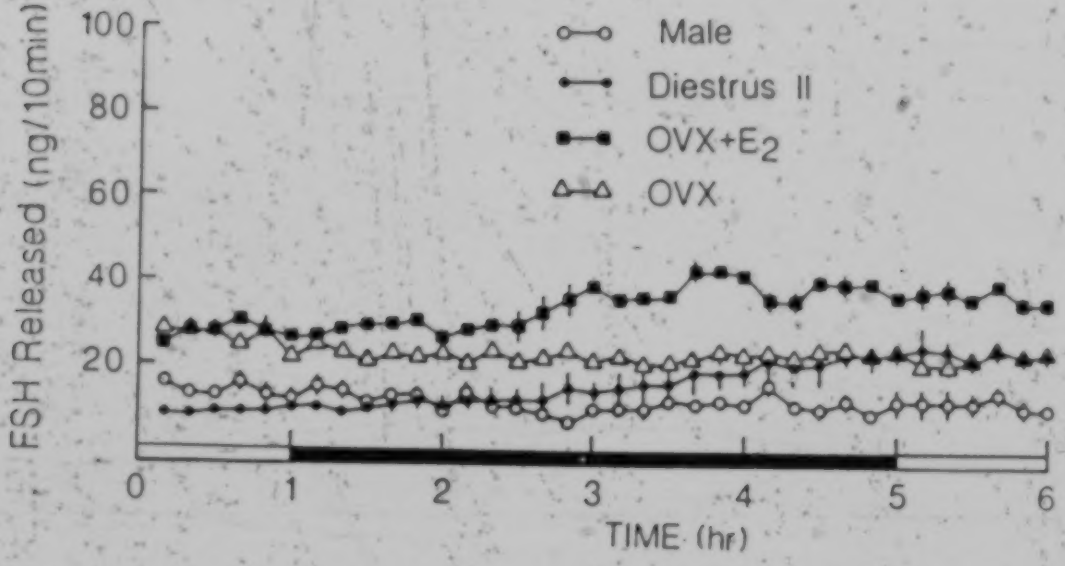

0.01 μM PMA
0.1 μM PMA
1 μM PMA
10 μM PMA
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

Fig. 3. Secretory profiles of LH and FSH obtained from pituitaries of intact males and diestrous II, OVX and OVX + E_2-treated females in response to continuous infusion (4 h) of 1 uM PMA (indicated by the black bar) in KIRB medium (n=3). For further details see legend for Fig. 2.


Male
Diestrus II
OVX+E2
OVX
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)


Male
Diestrus II
OVX+E2
OVX
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

pituitaries of DII females ($p < 0.01$, cf. Table 2). In contrast, the amounts of FSH secreted were not significantly different when the two groups were compared ($p > 0.05$, cf. Table 2).

Interestingly, the temporal characteristics of the secretory profiles obtained from pituitaries of diestrous II and OVX + E_2-treated females also differ. Increased gonadotropin secretion was evident within 30 min from pituitaries of OVX + E_2-treated females (Fig. 3) (circulating estradiol levels of 66 pg/mL, Appendix 1), while the onset of secretion from pituitaries of diestrous II females (circulating estradiol levels of 38 pg/mL, Appendix 1) was delayed for about 60 min (Fig. 3). A comparison of the amounts of LH secreted during the first hour, second hour and last 2 hours of PMA infusion is shown in Table 3. It is apparent that the ·aries from OVX + E_2-treated females secreted significantly greater ₁ of LH when compared to pituitaries from diestrous II females at each time period ($p < 0.01$).

The PMA-induced gonadotropin secretion from pituitaries of OVX + E_2 -treated and diestrous II females also appears to be dependent on de novo protein synthesis, in that the secretion was inhibited by 5 uM cycloheximide (Figs. 4 and 5).

Other reports had indicated that PMA can elicit LH secretion from primary anterior pituitary cells obtained from male rats (Smith and Vale, 1980; 1981). Given the fact that the PMA-induced secretion in the present study appears to be estradiol-dependent, it seemed plausible that the cultured pituitary cells obtained from male rats were responsive to PMA as a result of exposure to estradiol contained in the serum which was added to supplement the culture medium. Consequently, the following

TABLE 2.

Total amounts of LH and FSH released from the anterior pituitaries in response to continuous infusion (4 h) of 1 uM PMA in KIRB medium.

Experimental group	LH released (ng)*	FSH released (ng)*
Diestrous II rats	302±48	159±61
Ovariectomized rats	No response	No response
Ovariectomized + E_2-treated rats	980±129	189±26
Intact male rats	No response	No response
Orchidectomized rats	No response	No response
Orchidectomized + E_2-treated rats	387±41	119±36

* Values represent the mean ± SEM (n=3).

All the values are corrected for basal secretion.

For LH, the p-value between the secretion from pituitaries of DII and OVX + E_2-treated rats is <0.01 (ANOVA, see Materials and Methods).

For FSH, the p-value between the secretion from pituitaries of DII and OVX + E_2-treated rats is >0.05 .

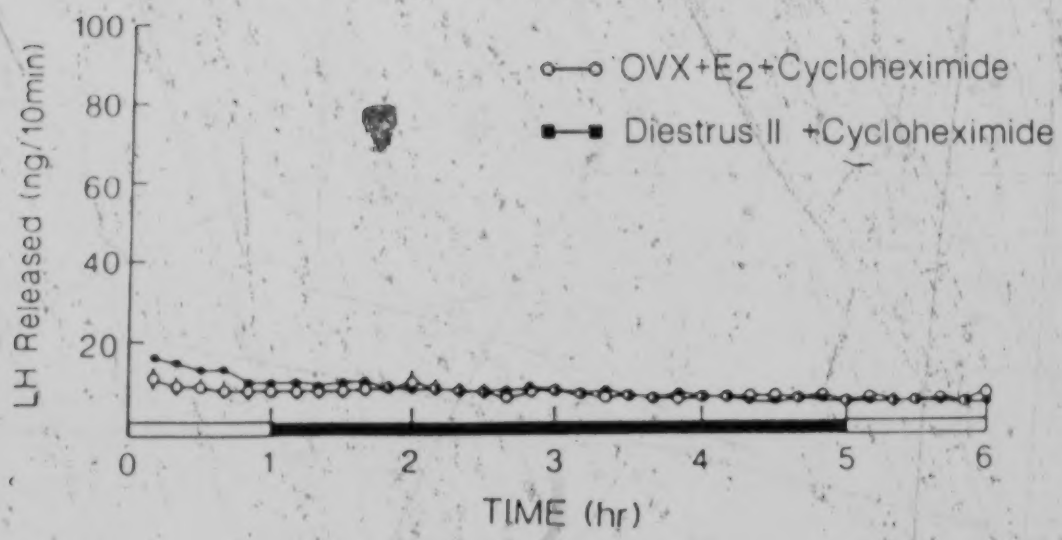
TABLE 3.

Total amounts of LH released from pituitaries of diestrous II and OVX + E_2-treated rats during the first, second and last two hours of continuous infusion of 1 uM PMA in KIRB medium.

Experimental group	LH released (ng)*		
	1st hour	2nd hour	Last 2 hours
DII females	0.06±1	38.2±3.5	263.8±12
OVX + E_2-treated	68.7±5	222.6±6	694.2±41

* Values represent the mean ± SEM. Results are corrected for basal secretion.

Fig. 4. Secretory profiles of LH and FSH obtained from pituitaries of diestrous II and OVX + E_2-treated females in response to continuous infusion (4 h) of 1 uM PMA (indicated by the black bar) in KIRB medium in the presence of cycloheximide (n=3). Cycloheximide was present in the medium at the time of collection of the pituitaries and throughout the experiment. For further details see legend for Fig. 2.


LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)
OVX+E2+Cycloheximide
Diestrus II +Cycloheximide

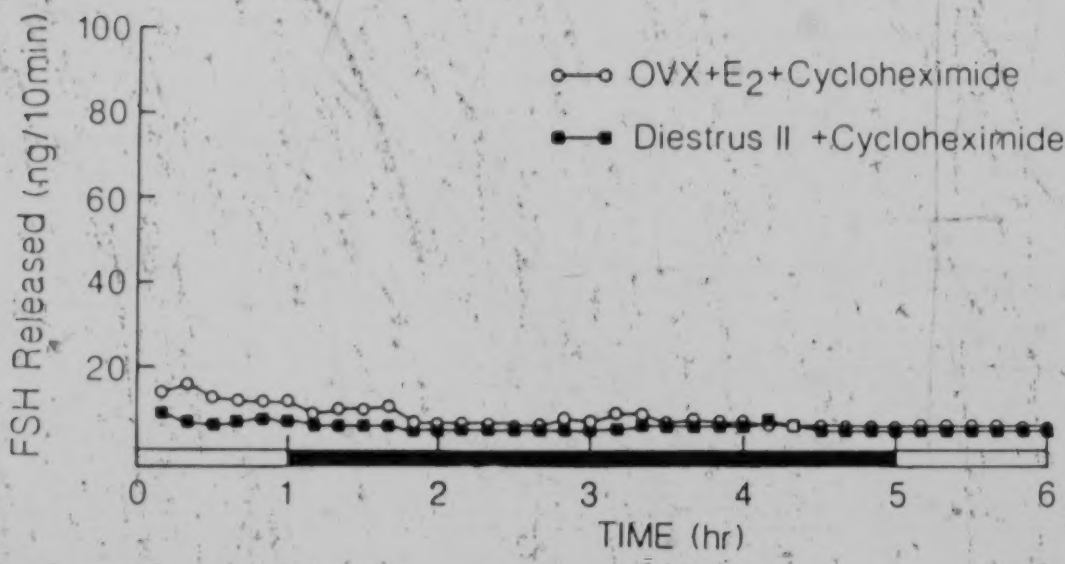
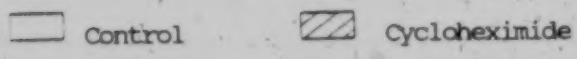
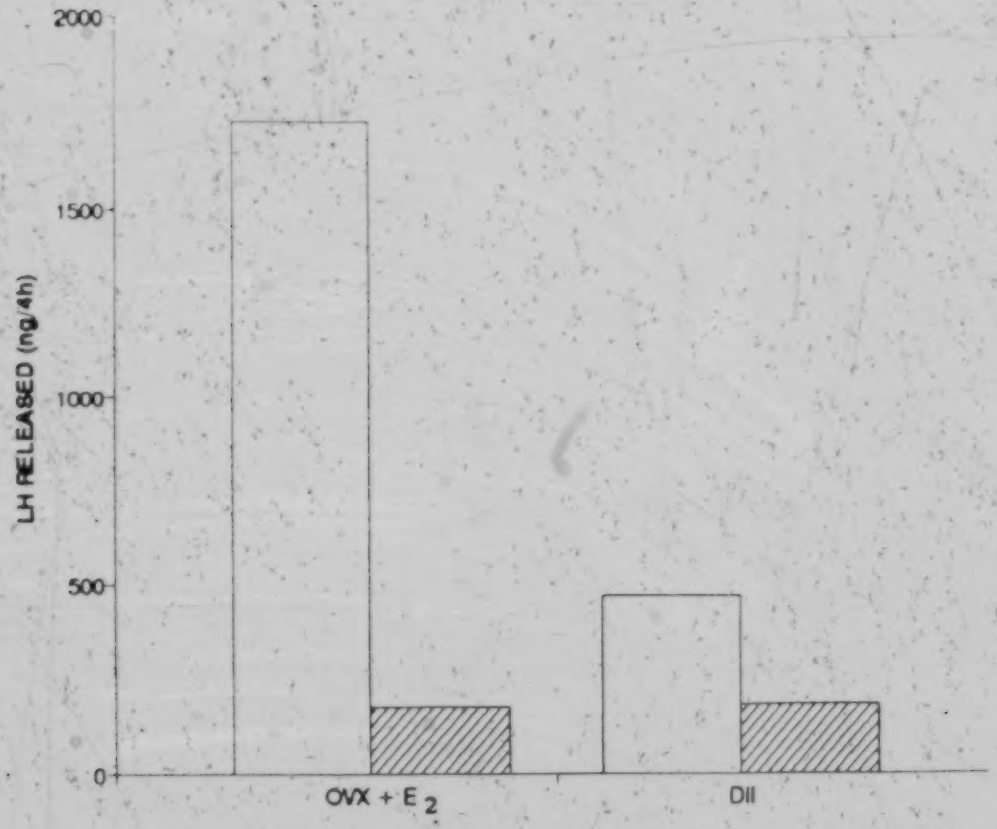
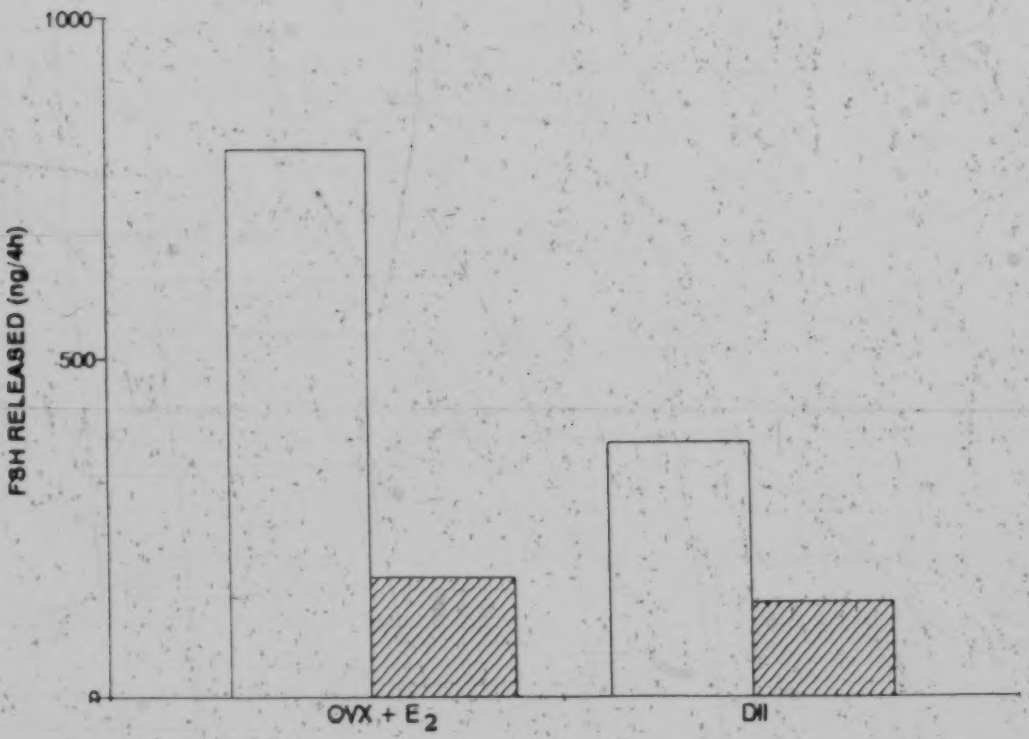

FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)
OVX+E2+Cycloheximide
Diestrus II +Cycloheximide

Fig. 5. Effect of cycloheximide on the total amounts of LH and FSH released from pituitaries of diestrous II (DII) and OVX + E_2-treated rats in response to continuous infusion (4 h) of 1 uM PMA in KIRB medium. The amounts of hormones were calculated as described in the Materials and Methods section without subtracting the basal values. Cycloheximide was present in the medium from the time of collection of the pituitaries and throughout the experiment. Data for statistical analysis appear in the text.




2000
1500
1000
500
0
LH RELEASED (ng/4h)
OVX + E2
DII


1000
500
0
FSH RELEASED (ng/4h)
OVX + E2
DII

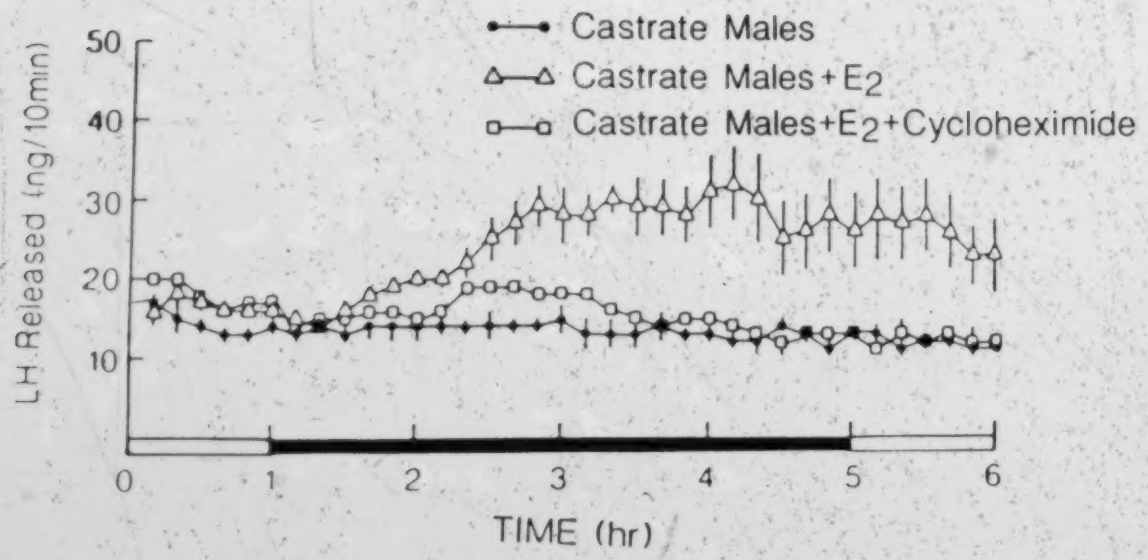
series of experiments were undertaken to determine whether exposure to estradiol would make gonadotrophs obtained from male rats responsive to PMA.

In the first experiment, adult males were castrated and half of the castrates were implanted subcutaneously (s.c.) with silastic capsules containing estradiol-benzoate 24 h prior to sacrifice. Since the dispersed cells would have been exposed to estradiol in the absence of testicular factors, castrated males were used to study the effects of estradiol, thereby removing the potential influences of testicular steroids and/or peptides. The results are shown in Fig. 6. As was the case with intact males, 1 uM PMA was ineffective in stimulating gonadotropin secretion from pituitaries of orchidectomized rats, while inducing the secretion of LH from pituitaries of orchidectomized + E_2 -treated rats. The FSH response was much less evident than the LH response. Furthermore, the PMA-induced gonadotropin secretion from pituitaries of castrated + E_2-treated males was inhibited by 5 uM cycloheximide, suggesting a dependency on de novo protein synthesis (Fig. 6).

In the second experiment, anterior pituitary cells dissociated from adult males were incubated in either Ham's F10 medium supplemented with horse serum and fetal calf serum, or Ham's F10 medium supplemented with intact male rat serum. The results are shown in Fig. 7. As expected, the cells incubated in the medium supplemented with horse serum and fetal calf serum were responsive to PMA ($p < 0.01$). Surprisingly, however, the cells incubated with intact male rat serum were also responsive to PMA ($p < 0.05$), although the response was attenuated when compared to the

response obtained from the cells grown in presence of horse and fetal calf sera ($p < 0.05$).

Fig. 6. Secretory profiles of LH and FSH obtained from pituitaries of castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 uM PMA (indicated by the black bar) in KIRB medium in the presence or absence of cycloheximide (n=3). Cycloheximide was present in the medium at the time of collection of the pituitaries and throughout the experiment. For further details see legend for Fig. 2.


Castrate Males
Castrate Males + E2
Castrate Males + E2 + Cycloheximide
LH Released (ng/10min)
50
40
30
20
10
0
1
2
3
4
5
6
TIME (hr)

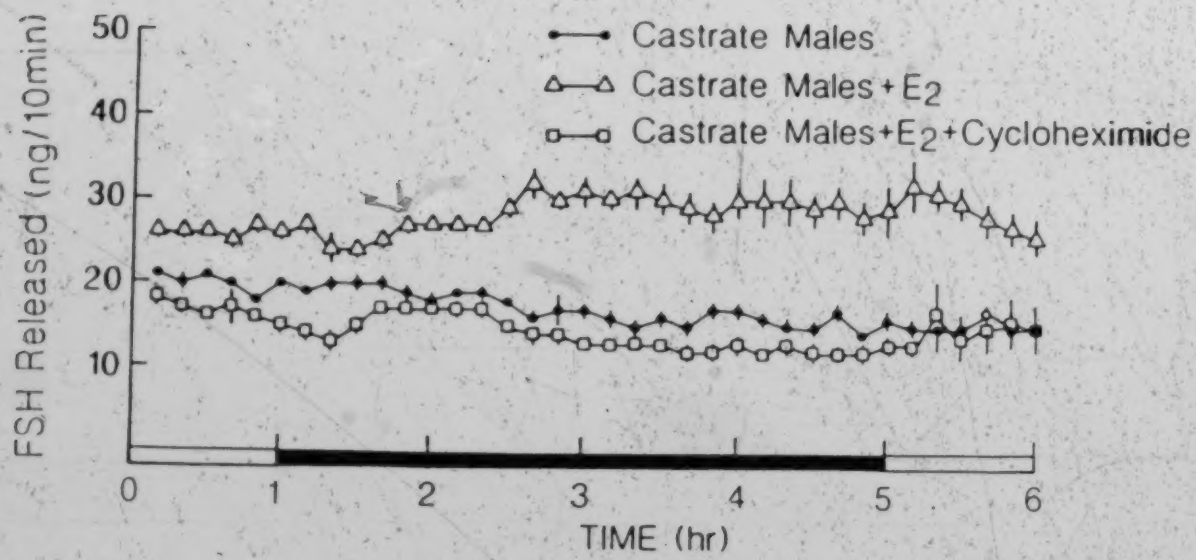
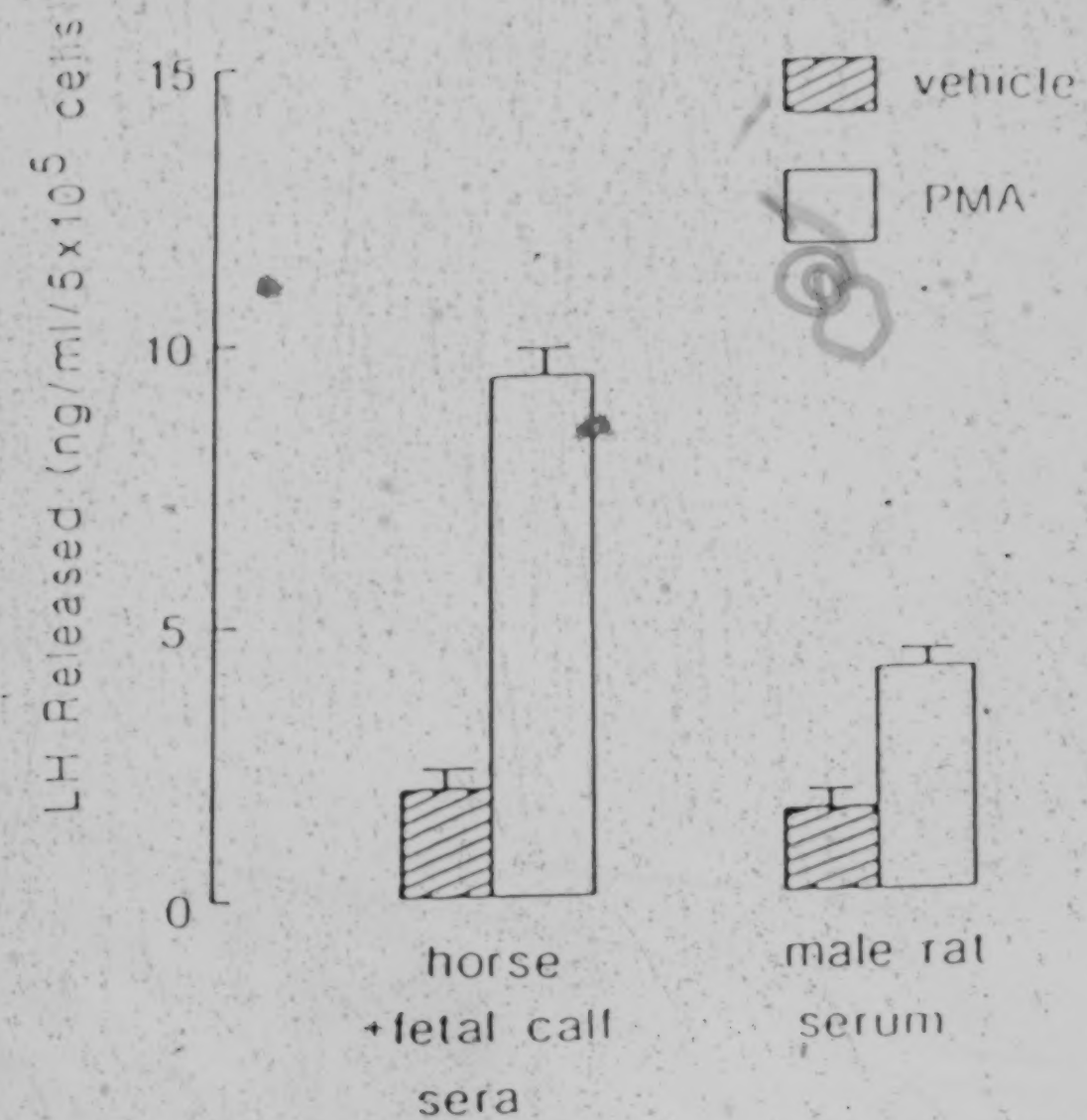

Castrate Males
Castrate Males + E2
Castrate Males + E2 + Cycloheximide
FSH Released (ng/10min)
50
40
30
20
10
0
1
2
3
4
5
6
TIME (hr)

Fig. 7. LH responses to 1 uM PMA (4 h) obtained from 5×10^5 dispersed anterior pituitary cells from male rats incubated in medium supplemented with either horse and fetal calf sera or male rat serum (n=5). See text for statistical analysis.

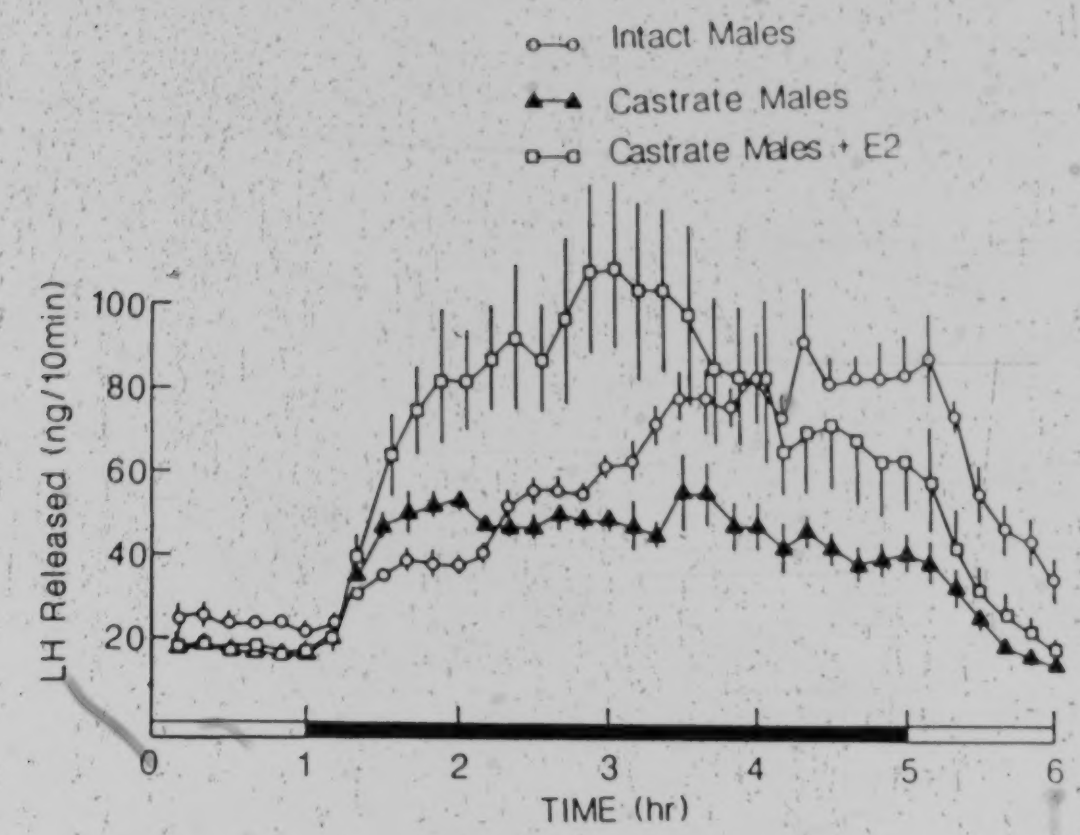

LH Released (ng/ml/5x10⁵ cells)
15
10
5
0
vehicle
PMA
horse
+fetal calf
sera
male rat
serum

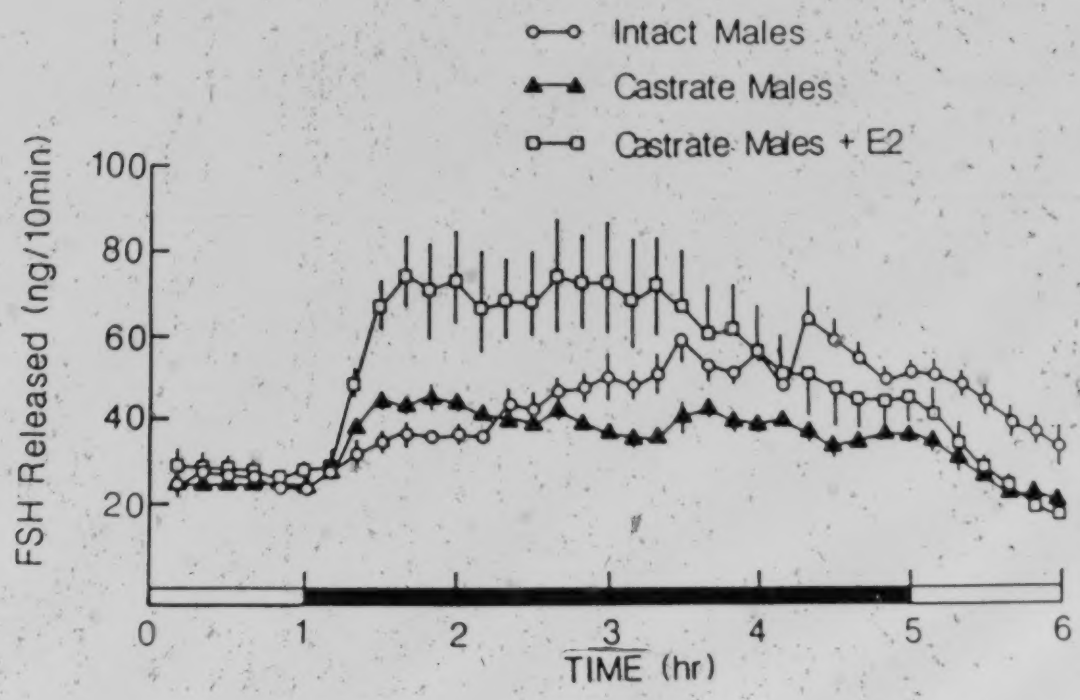
II. Induction of an Extracellular Calcium-Independent Component of Gonadotropin Secretion from the Anterior Pituitaries of Male Rats.

Previous studies from this laboratory had demonstrated the manifestation of a GnRH-stimulated extracellular calcium-independent component of gonadotropin secretion from pituitaries of females but not males (Bourne et al., 1988; Bourne, 1988). This component of gonadotropin secretion was shown to be dependent on estradiol. Subsequent studies demonstrated the ability of PMA to mimic the characteristics of the GnRH-stimulated extracellular calcium-independent secretion of gonadotropins (Bourne et al., 1989), suggesting that the PMA-induced mechanisms might be similar to those mediating the GnRH-stimulated responses. Since the initial study in this report indicated a dependency of PMA-stimulated gonadotropin secretion on estradiol, the present study was undertaken to determine whether estradiol could induce a PMA- and/or GnRH-stimulated extracellular calcium-independent secretion of LH and FSH from pituitaries of castrated males.

The first series of experiments was undertaken to characterize the secretory profiles of gonadotropins obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to GnRH in KIRB medium. The results are shown in Fig. 8. Continuous infusions of 1 nM GnRH to pituitaries from intact male rats resulted in a slow but steady increase in LH secretion for three hours, at which time the secretory rate stabilized until infusion of the decapeptide was terminated. The response from pituitaries of castrated males exhibited an initial steeper increase ($p < 0.05$) in gonadotropin secretion with the secretory rate

Fig. 8. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 nM GnRH (indicated by the black bar) in KIRB medium (n=3). For further details see legend for Fig. 2.


Intact Males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)


Intact Males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

remaining relatively constant after 30 min of GnRH infusion. This analysis of data was performed by comparing the amount of LH secreted for each individual hour of the GnRH infusion and will be presented with the ionomycin data in Table 8. In spite of the lower secretion rate from pituitaries of castrated males, the total amount of LH secreted from pituitaries of castrated males was not significantly different from that obtained from pituitaries of intact males ($p > 0.05$). The responses from the pituitaries obtained from castrated + E_2-treated male rats were much greater than those obtained from either intact, or castrated male pituitaries for the first 2 hours of GnRH admininstration ($p < 0.01$). After 2 h, the secretion of LH started to decline inspite of the continued infusion of GnRH. The secretory profiles of FSH obtained in response to GnRH were qualitatively similar to those for LH, although the amounts of FSH secreted were lower than those of LH (Fig. 8 and Table 4).

The effects of cycloheximide on GnRH-stimulated gonadotropin secretion are shown in Fig. 9. Cycloheximide inhibited a major component of the GnRH-stimulated responses in all three treatment groups (cf. Table 5). Interestingly, the amounts of LH and FSH secreted from the pituitaries obtained from intact, castrated and castrated + E_2-treated males were similar in the presence of the protein synthesis inhibitor (Table 5).

As expected, GnRH was effective in stimulating gonadotropin secretion from pituitaries obtained from castrated + E_2-treated males in the absence of extracellular calcium (Fig. 10). Surprisingly, however, the decapeptide also stimulated a low level of LH and FSH secretion from pituitaries of castrated and intact males. In contrast to the delayed secretion obtained from pituitaries of females (cf. Bourne et al., 1989),

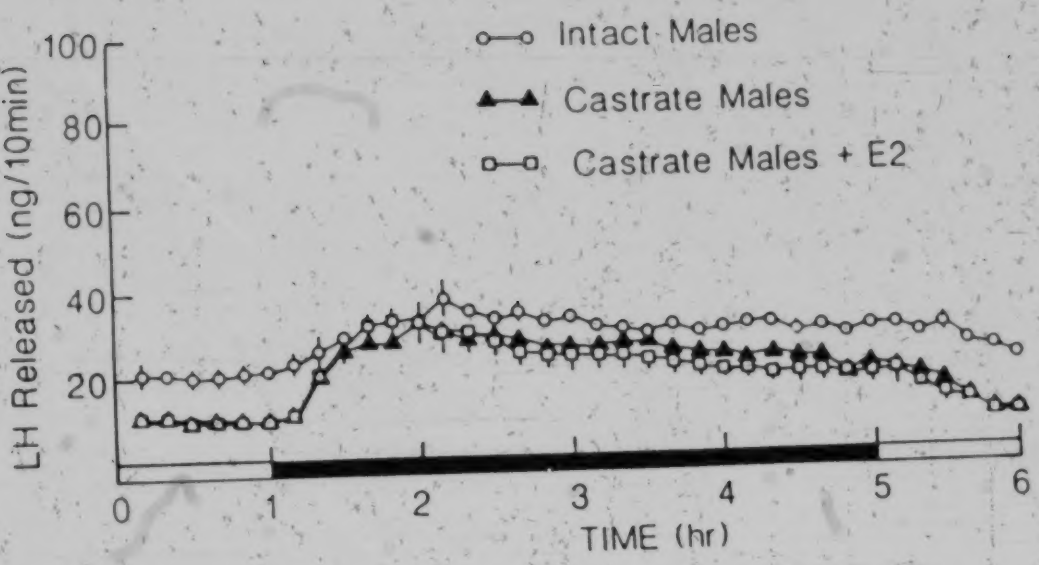
TABLE 4.

Total amounts of LH and FSH released from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 nM GnRH in KIRB or calcium-free medium.

Experimental Group	KIRB medium		Calcium-free medium	
	LH	FSH	LH	FSH
	(ng)*		(ng)*	
Intact males	796±15	504±70	383±12	384±17
Castrated males	683±128	312±23	309±12	100±16
Castrated + E_2-treated males.	1490±439	778±257	931±4	526±12

* The values represent the mean ± SEM (n=3).
All values were corrected for basal secretion.

Fig. 9. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 nM GnRH (indicated by the black bar) in KIRB medium in the presence of cycloheximide (n=3). Cycloheximide was present in the medium from the time of collection of the pituitaries and throughout the experiment. For further details see legend for Fig. 2.


Intact Males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

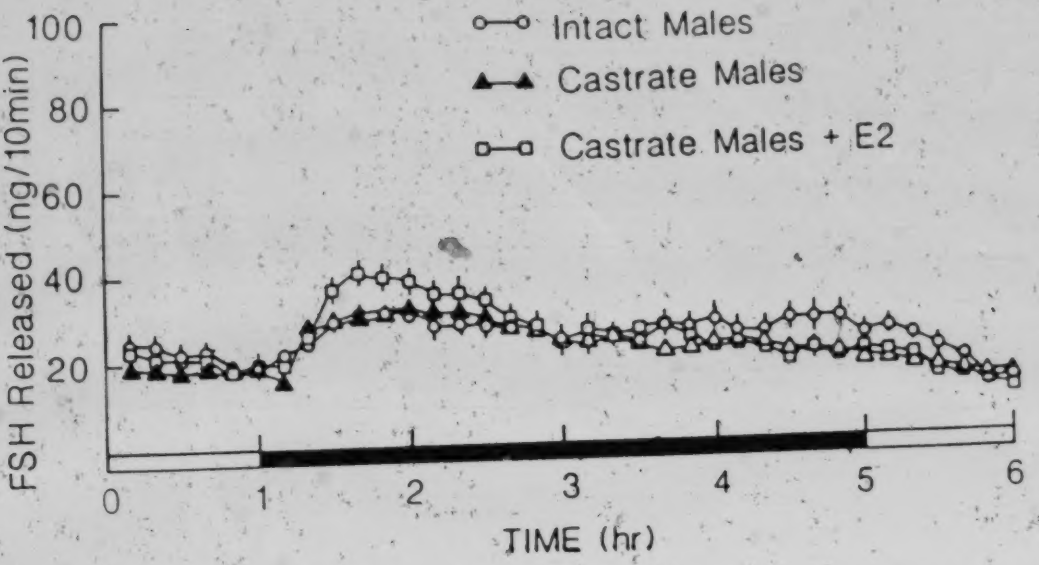
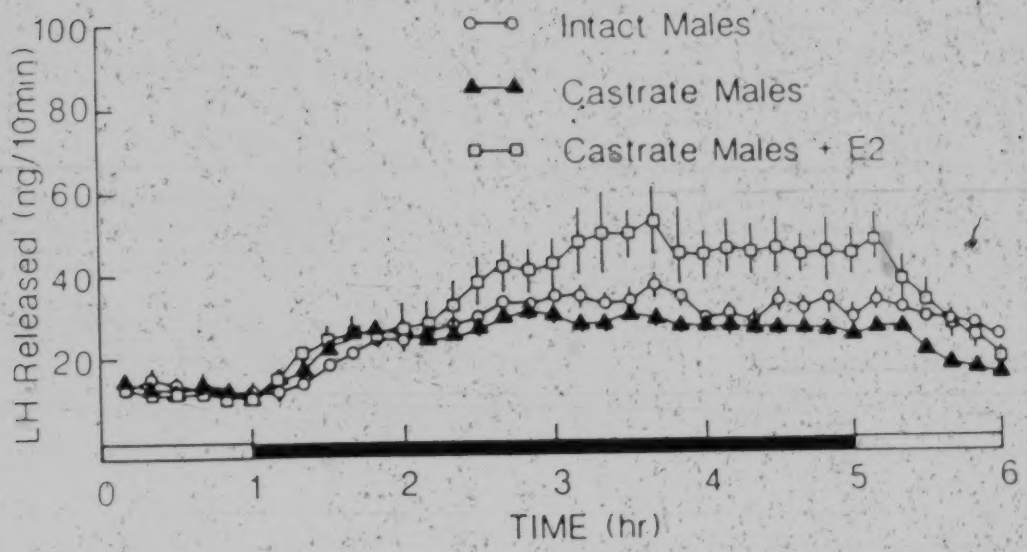
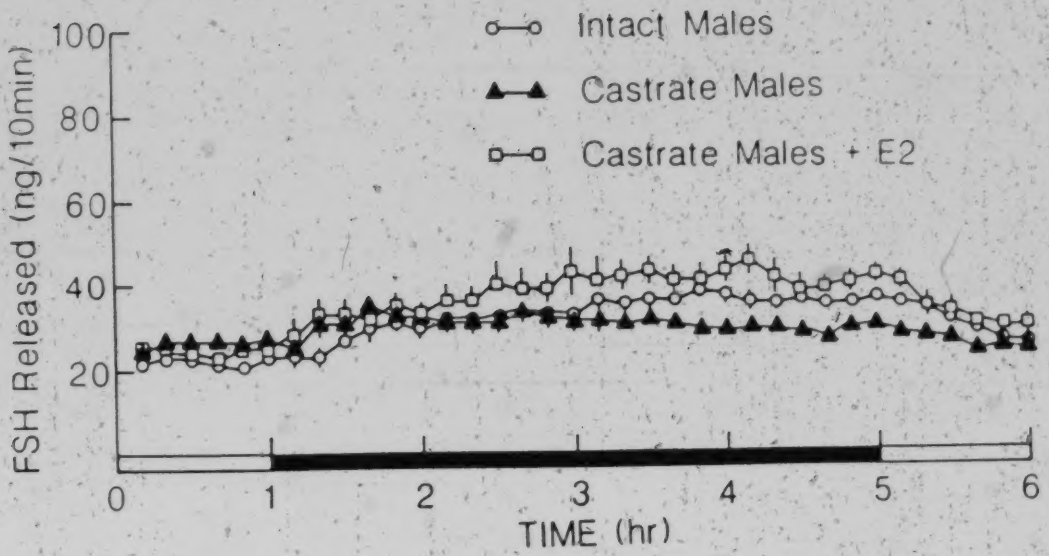

Intact Males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

Fig. 10. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 nM GnRH (indicated by the black bar) in calcium-free medium (n=3). For further details see legend for Fig. 2.


Intact Males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

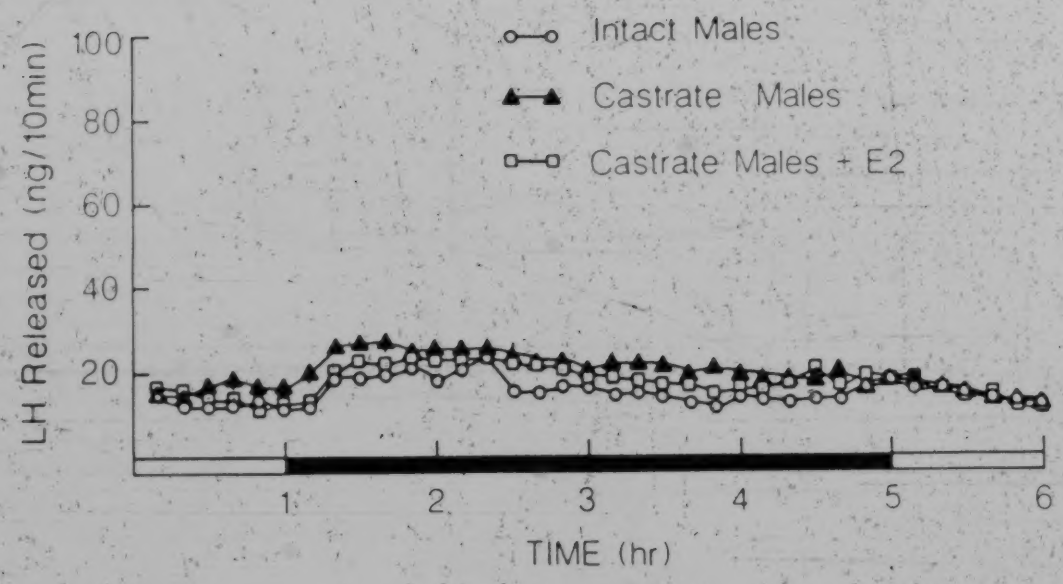

Intact Males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

the low secretion from pituitaries of males had a rapid onset. Thus, the responses from all three groups of pituitaries were similar within the first hour of GnRH administration (Fig. 10). However, after approximately 70 minutes, the pituitaries from castrated + E_2-treated males exhibited an increased response which was maintained until the infusion of the decapeptide was terminated (Fig. 10). It is noteworthy that the GnRH-stimulated gonadotropin secretion from pituitaries of intact, castrated or castrated + E_2-treated males perifused with calcium-free medium is much lower than the corresponding responses obtained in KIRB medium (Table 4).

To determine whether de novo protein synthesis is required for the GnRH-stimulated extracellular calcium-independent component of gonadotropin secretion, the previous experiment was repeated in the presence of cycloheximide. The results are shown in Fig. 10 and Table 6. The responses from the pituitaries of intact, castrated and castrated + E_2-treated males were all affected by cycloheximide (Table 6). However, inhibition of secretion was greatest in the pituitaries obtained from castrated + E_2-treated males. As a result, the responses from the pituitaries of intact, castrated or castrated + E_2-treated males were similar to each other, indicating that part of the the low, rapid secretion was independent of de novo protein synthesis, whereas the estradiol-induced enhanced response was not.

Subsequent experiments were performed to determine whether PMA could mimic the characteristics of the GnRH-stimulated extracellular calcium-independent responses from pituitaries of male rats. The results are shown in Fig. 12. Pituitaries obtained from intact or castrated

Fig. 11. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 nM GnRH (indicated by the black bar) in calcium-free medium in the presence of cycloheximide (n=3). Cycloheximide was present in the medium from the time of collection of the pituitaries and throughout the experiment. For further details see legend for Fig. 2.


Intact Males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
1
2
3
4
5
6
TIME (hr)

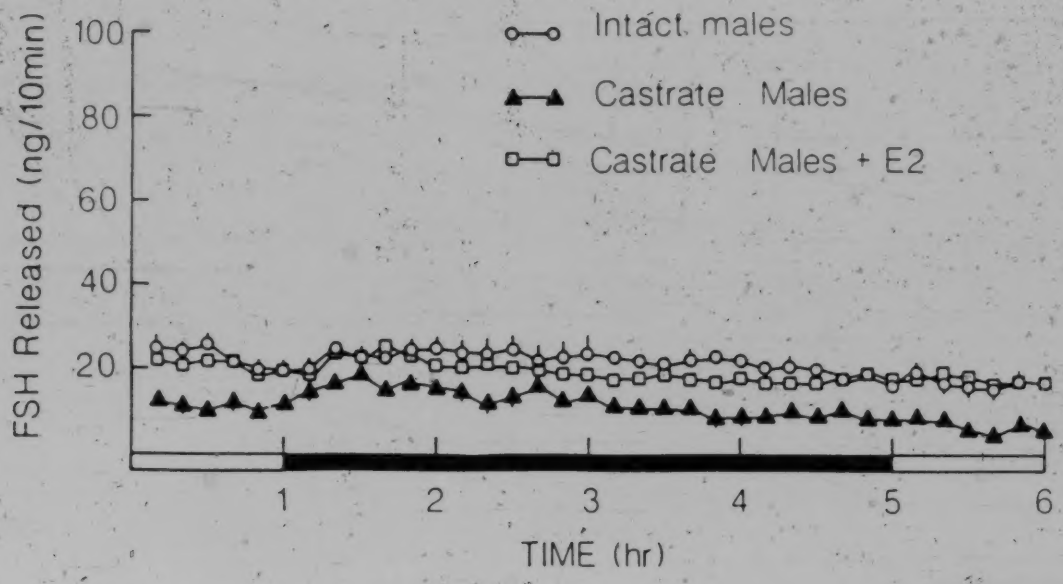
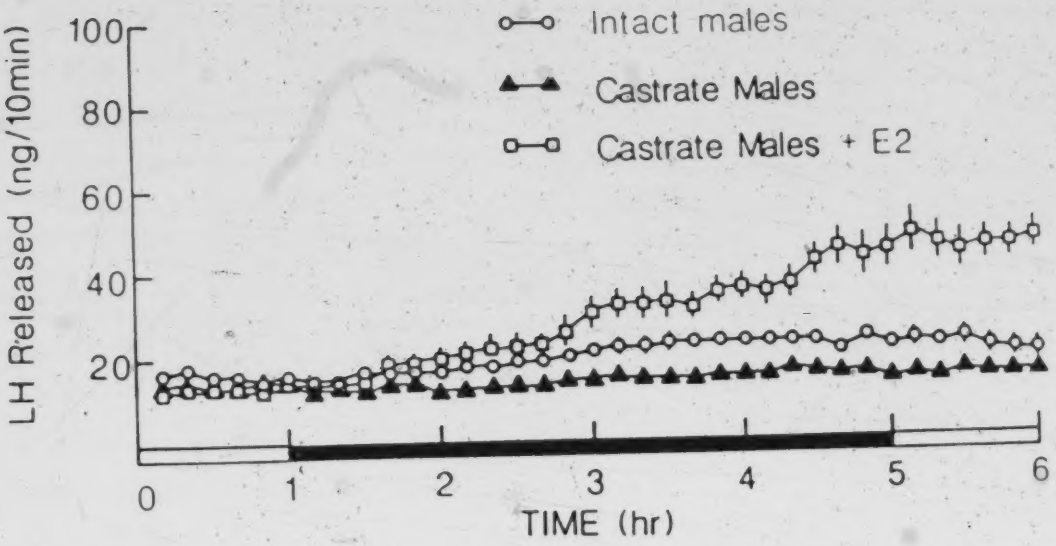

Intact males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
1
2
3
4
5
6
TIME (hr)

Fig. 12. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 uM PMA (indicated by the black bar) in calcium-free medium (n=3). For further details see legend for Fig. 2.


Intact males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

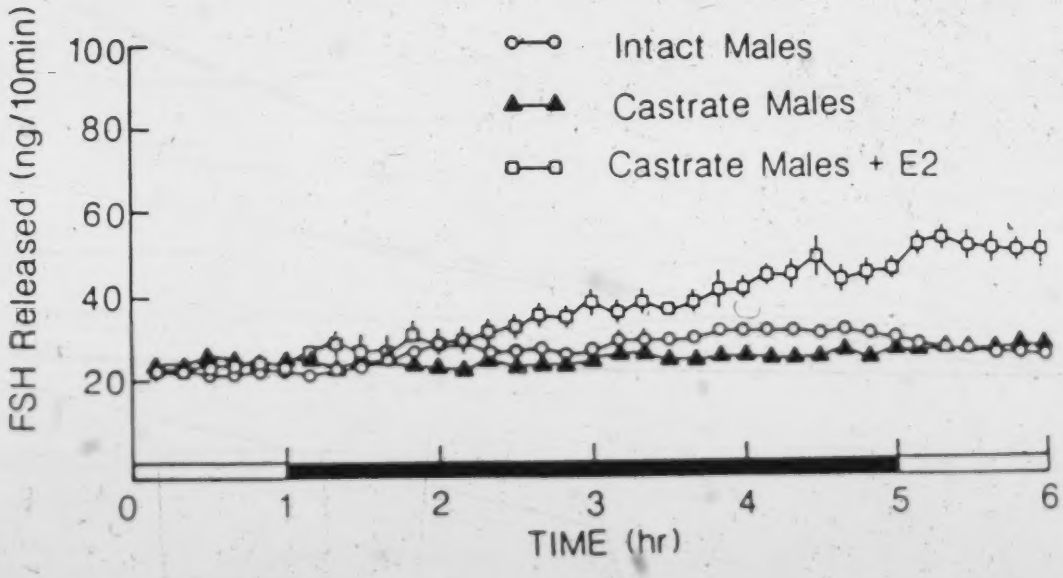

Intact Males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

TABLE 5.

Total amounts of LH and FSH released from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 nM GnRH in KIRB medium in the presence or absence of cycloheximide.

Experimental group	LH (ng)*			FSH (ng)*		
	KIRB	KIRB+cycloh.	p-value	KIRB	KIRB+Cycloh.	p-value
Intact males	796±15	270±78	p<0.01	504±70	242±8	p<0.01
Castrated males	683±128	359±28	p<0.05	312±23	179±36	p<0.05
Castrated + E_2 males	1490±439	325±67	p<0.01	778±257	181±37	p<0.05

* Values represent the mean ± SEM. All values were corrected for basal secretion.

TABLE 6.

Total amounts of LH and FSH released from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 1 nM GnRH in calcium-free medium in the presence or absence of cycloheximide.

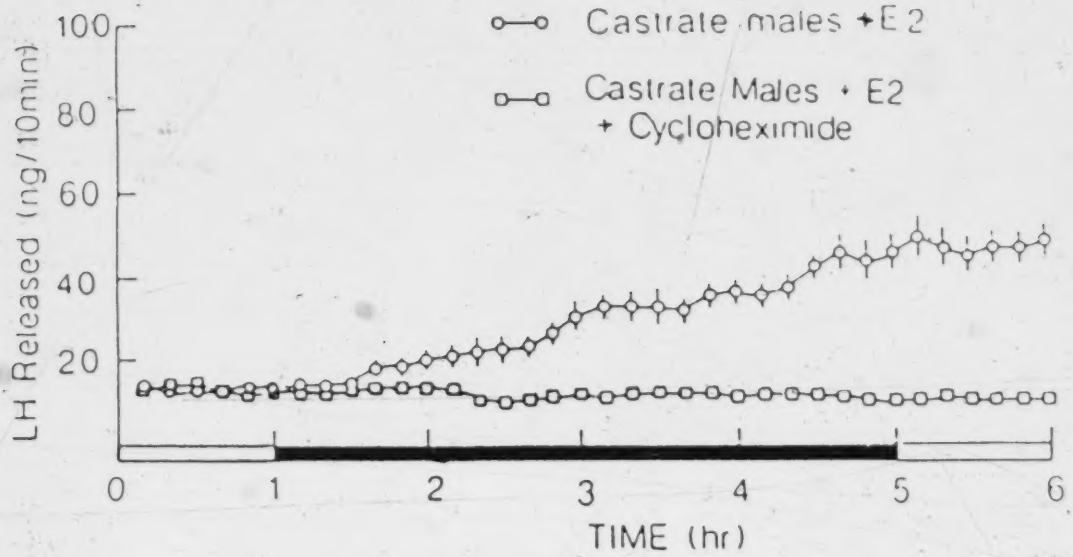
Experimental group	LH (ng)*			FSH (ng)*		
	Calcium-free	Calcium-free+cycloh.	p-value	Calcium-free	Calcium-free+cycloh.	p-value
Intact males	383±12	72±34	p<0.01	384±17	27±14	p<0.01
Castrated males	310±12	132±18	p<0.01	100±16	38±9	p<0.01
Castrated + E_2 males	931±4	125±64	p<0.001	526±12	28±3	p<0.001

* Values represent the mean ± SEM. All values were corrected for basal secretion.

males did not respond to continuous infusions of 1 uM PMA, and hence did not duplicate the GnRH-stimulated response. In contrast, pituitaries obtained from castrated + E_2-treated male rats responded to PMA with an increased secretion of gonadotropins that was evident approximately 40-60 min after the beginning of PMA infusions (Fig. 12). The PMA-stimulated gonadotropin secretion obtained from pituitaries of castrated + E_2-treated males was inhibited by cycloheximide (Fig. 13 and Table 7).

Taken together, the results suggest that the PMA-stimulated secretory mechanisms are not associated with the low, rapid GnRH-stimulated extracellular calcium-independent responses. However, it would appear that the PMA-stimulated mechanisms may account for the enhanced response obtained from the pituitaries of castrated + E_2-treated male rats; i.e. the estradiol-induced enhanced response might be associated with mechanisms that can be stimulated by PMA and are dependent on de novo protein synthesis.

Fig. 13. Secretory profiles of LH and FSH obtained from pituitaries of castrated + E_2-treated males in response to continuous infusion (4 h) of 1 uM PMA (indicated by the black bar) in calcium-free medium in the presence or absence of cycloheximide (n=3). Cycloheximide was present in the medium from the time of collection of the pituitaries and throughout the experiment. For more details, refer to legend for Fig. 2.


Castrate males + E 2
Castrate Males + E2
+ Cycloheximide
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

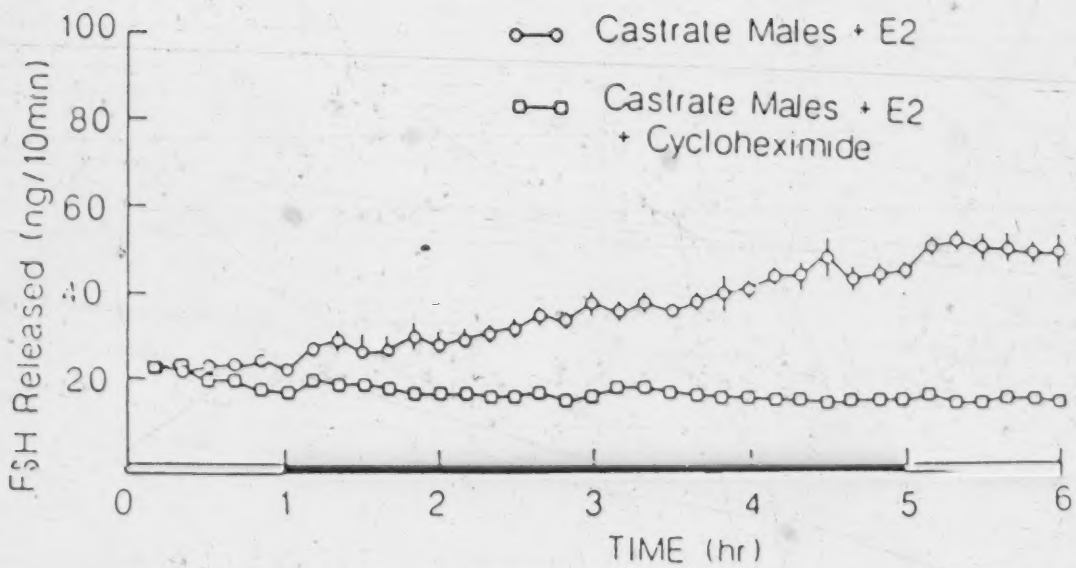

Castrate Males + E2
Castrate Males + E2
+ Cycloheximide
FSH Released (ng/10min)
100
80
60
20
0
1
2
3
4
5
6
TIME (hr)

TABLE 7.

Total amounts of LH and FSH released from pituitaries of castrated + E_2 -treated males in response to continuous infusion (4 h) of 1 uM PMA in calcium-free medium in the presence or absence of cycloheximide .

	- cycloheximide	+ cycloheximide	p-value
LH secreted (ng)*	611±40	264±13	$p<0.01$
FSH secreted (ng)*	862±32	396±7	$p<0.01$

* Values represent the mean ± SEM (n = 3).

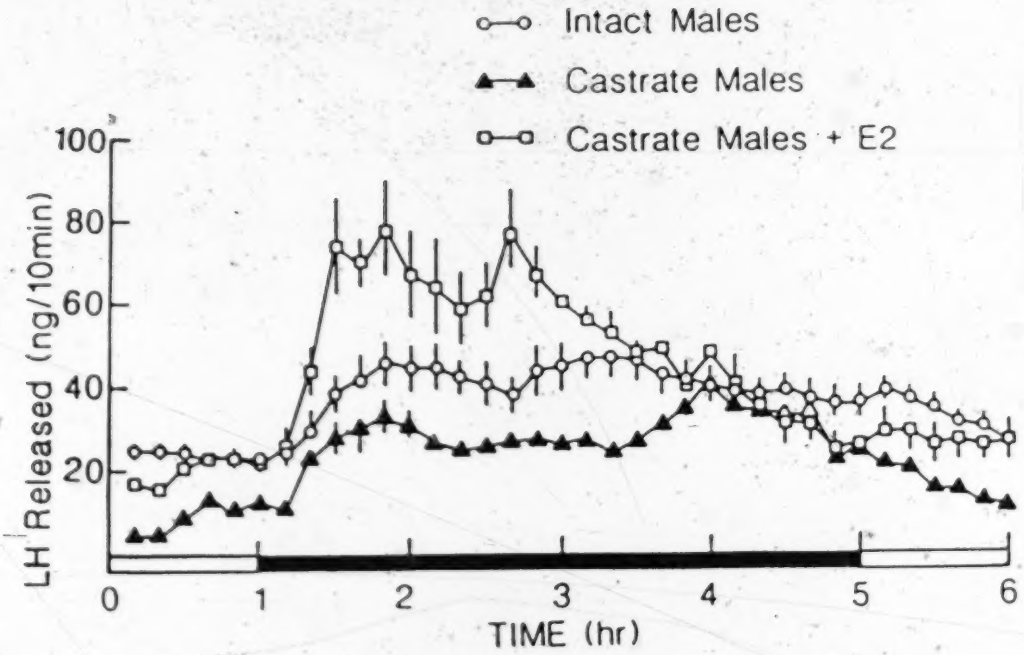
Values were <u>not</u> corrected for basal secretion, since this would have resulted in negative values.

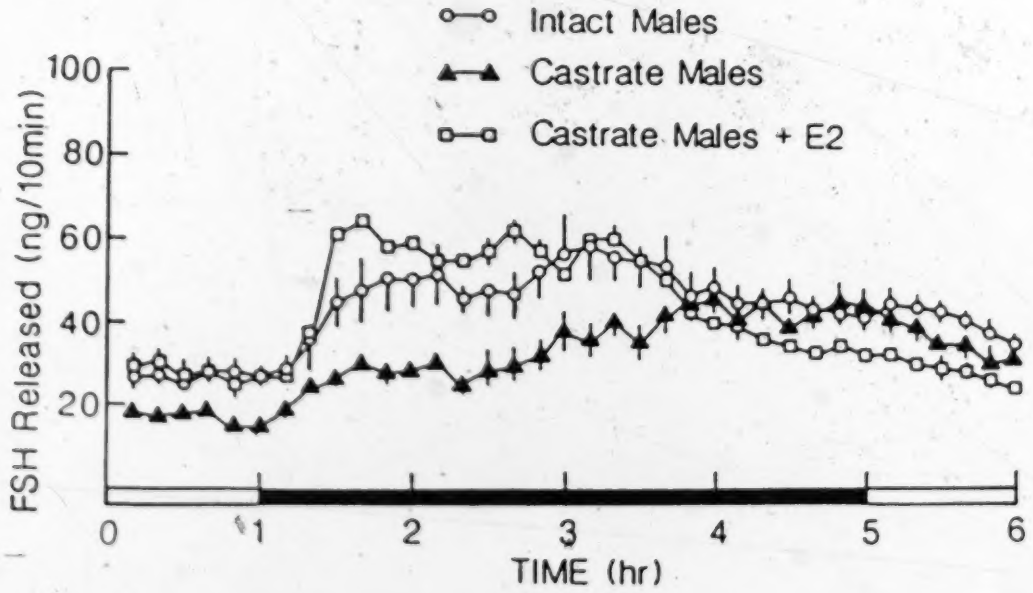
III. Interactions Between the Secretory Mechanisms Activated by Ionomycin and PMA in the Anterior Pituitaries of Male Rats.

In the preceding studies, the ability of PMA to stimulate the release of gonadotropins from pituitaries of male rats in the presence or absence of extracellular calcium was shown to be estradiol-dependent. Since several reports had documented synergistic interactions between calcium-mobilizing agents and protein kinase C activators (Naor and Eli, 1985; Wooge and Conn, 1987; Das et al., 1989), additional studies were conducted to investigate a potential estradiol-dependency of the synergistic interactions between these two systems.

The first series of experiments was performed to characterize the secretory profiles obtained from pituitaries of male rats in response to the calcium mobilizing agent, ionomycin. Pituitaries obtained from the three treatment groups (intact, castrated and castrated + E_2-treated males) were simultaneously perifused with 10 uM ionomycin. The concentration of ionomycin was chosen according to a dose-response curve that was reported previously from this laboratory (Das et al., 1989). The results are shown in Fig. 14. The secretory responses to ionomycin from intact and castrated male pituitaries were qualitatively similar. These responses were characterized by a rapid onset of secretion which stabilized approximately 40 min after ionomycin administration, and remained at that level for the duration of the experimental period. In sharp contrast to these results, the pituitaries from castrated + E_2 -treated males exhibited a much greater initial response which started to decrease after 2 h of ionomycin infusion (Fig. 14). The amount of hormone secreted in the last hour of ionomycin infusion was significantly

Fig. 14. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 10 uM ionomycin (indicated by the black bar) in KIRB medium (n=3). For further details see legend for Fig. 2.


Intact Males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

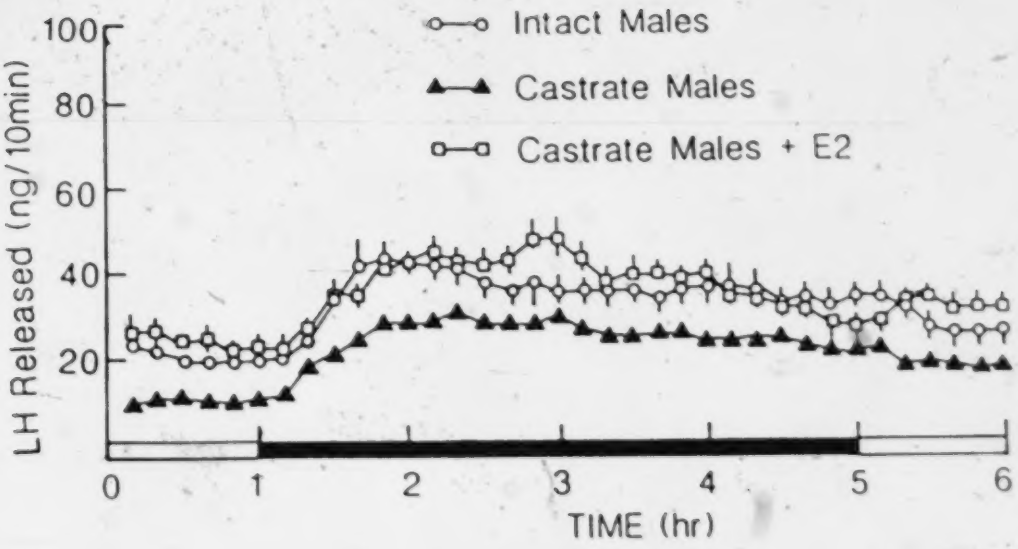

Intact Males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

reduced when compared to the previous 3 hours ($p < 0.05$) (cf. Table 8).A similar effect was observed when pituitaries of castrated + E_2-treated males were infused with GnRH in KIRB medium (cf. Fig. 8).

The ionomycin-induced gonadotropin secretory profiles obtained from pituitaries of intact or castrated males were not affected by cycloheximide (Fig. 15). However, the compound did inhibit the enhanced response obtained from the pituitaries of castrated + E_2-treated males ($p < 0.05$), resulting in a secretory profile which was qualitatively (Fig. 15), and quantitatively (Fig. 16) similar to those obtained from pituitaries of intact and castrated male rats ($p > 0.05$). Thus, the secretion of LH and FSH obtained from pituitaries of males in response to ionomycin appears to be independent of de novo protein synthesis, except when the pituitaries are exposed to estradiol (i.e. the estradiol-induced enhanced response is dependent on de novo protein synthesis).

The final series of experiments was designed to investigate potential interactions between the calcium-mobilizing agent and PMA-activated mechanisms. To accomplish this, pituitaries obtained from intact, castrated and castrated + E_2-treated males were simultaneously perifused with PMA and ionomycin together. The results are shown in Fig. 17. Interestingly, the responses from the pituitaries obtained from intact, castrated and castrated + E_2-treated males were qualitatively similar, with the responses from the pituitaries of castrated + E_2-treated males not showing the decline in secretion observed when ionomycin was infused alone. The total amount of gonadotropins secreted in response to the simultaneous administration of ionomycin and PMA was greater than the sum of the hormones released in response to the individual secretagogues

Fig. 15. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 10 uM ionomycin (indicated by the black bar) in KIRB medium in the presence of cycloheximide (n=3). Cycloheximide was present in the medium from the time of collection of the pituitaries and throughout the experiment. For further details see legend for Fig. 2.


Intact Males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

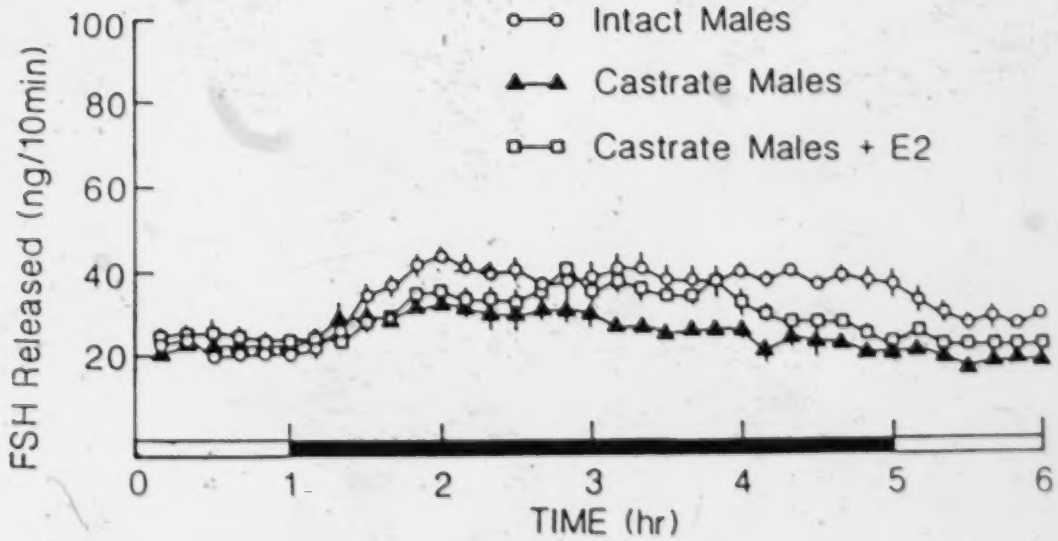
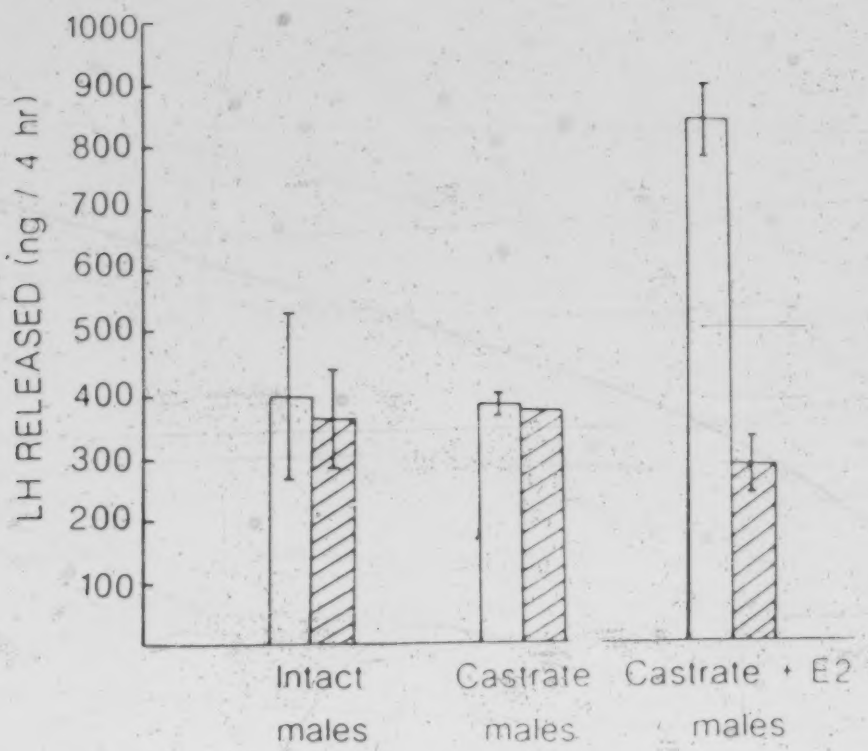

Intact Males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

Fig. 16. Effect of cycloheximide on the total amounts of LH and FSH released from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 10 uM ionomycin in KIRB medium. The amounts of the hormones were calculated as described in the Materials and Methods section. Cycloheximide was present in the medium from the time of collection of the pituitaries and throughout the experiments.


LH RELEASED (ng / 4 hr)
1000
900
800
700
600
500
400
300
200
100
Intact males
Castrate males
Castrate + E2 males

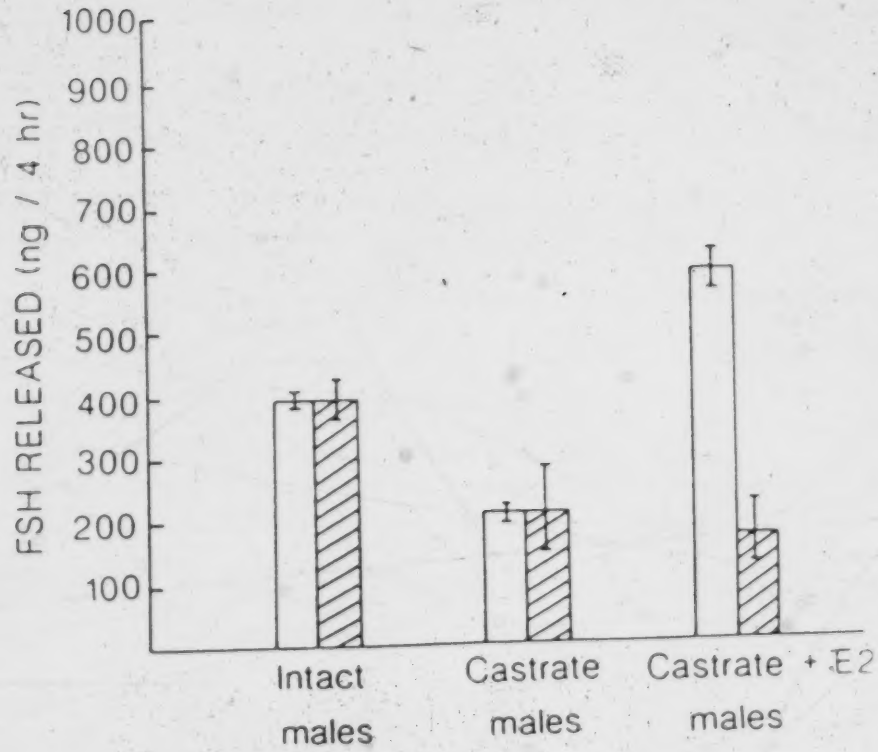
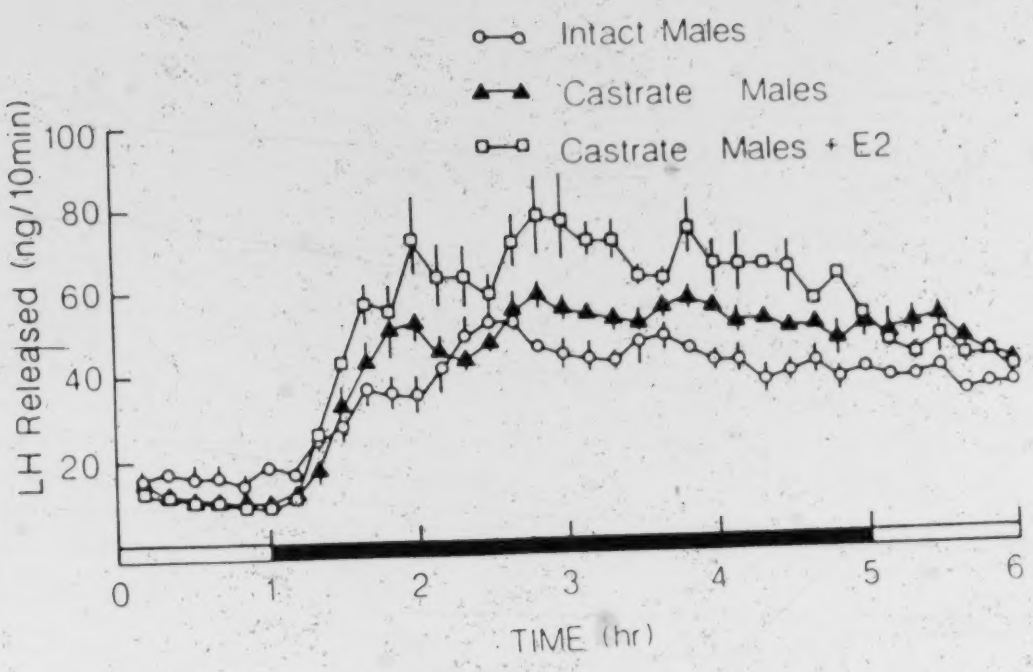

FSH RELEASED (ng / 4 hr)
1000
900
800
700
600
500
400
300
200
100
Intact males
Castrate males
Castrate + E2 males

Fig. 17. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 10 uM ionomycin and 1 uM PMA simultaneously (indicated by the black bar) in KIRB medium (n=3). For further details see legend for Fig. 2.


Intact Males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

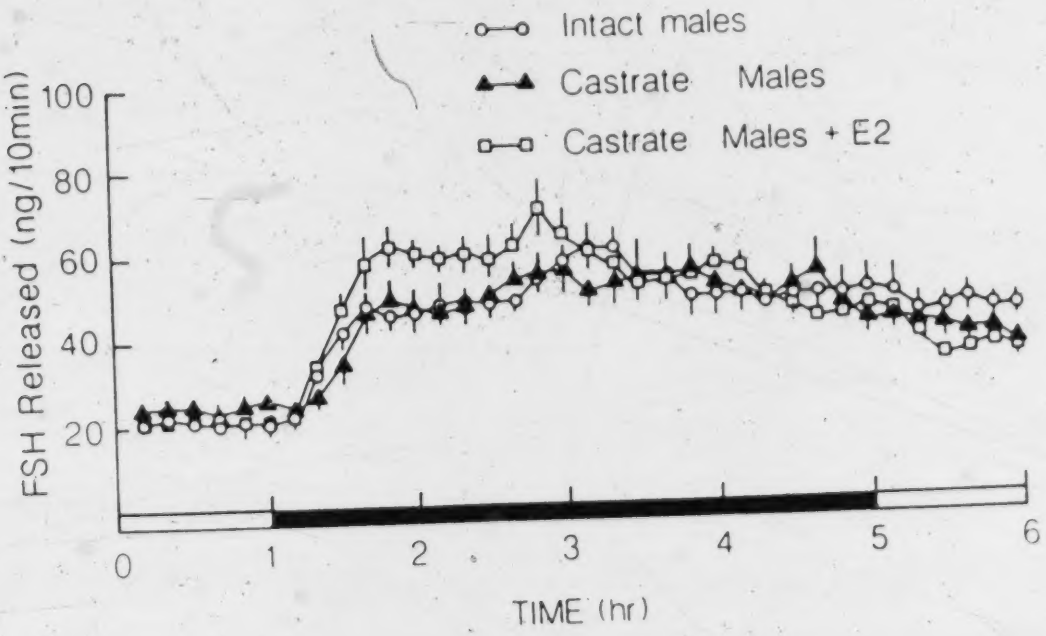

Intact males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

TABLE 8.

Total amounts of LH and FSH secreted from pituitaries of castrated + E_2 -treated males during the first, second, third and fourth hours of continuous infusion (4 h) of 1 nM GnRH or 10 uM ionomycin in KIRB medium.

Secretagogue	LH (ng)*				FSH (ng)*			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	4 h
GnRH	259±25	476±53	454±58	318±42	193±18	255±23	217±31	113±22
Ionomycin	204±4	259±13	244±10	159±3	142±9	173±7	140±6	43±9

* Values represent the mean ± SEM. All values were corrected for basal secretion.

TABLE 9.

Total amounts of LH and FSH released from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusions (4 h) of various secretagogues in KIRB medium.

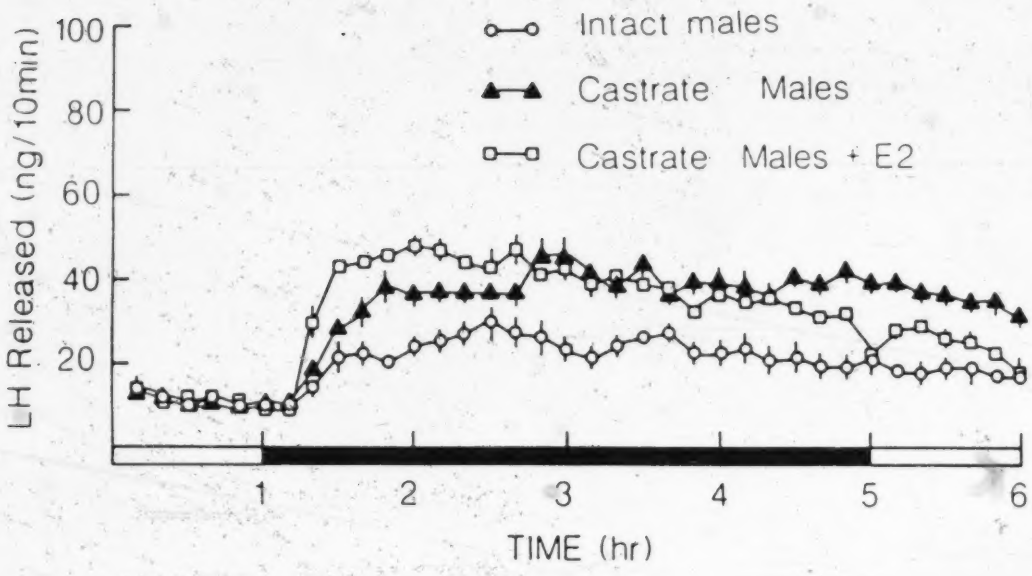
Secretagogues	Experimental Groups					
	Intact males		Castrated males		Castrated + E_2 males	
	LH	FSH	LH	FSH	LH	FSH
	(ng)*		(ng)*		(ng)*	
Ionomycin	399±100	388±12	357±18	224±10	866±65	576±38
PMA	0	0	0	0	388±50	119±35
Ionomycin + PMA	580±54	571±42	880±90	563±89	1246±89	770±115

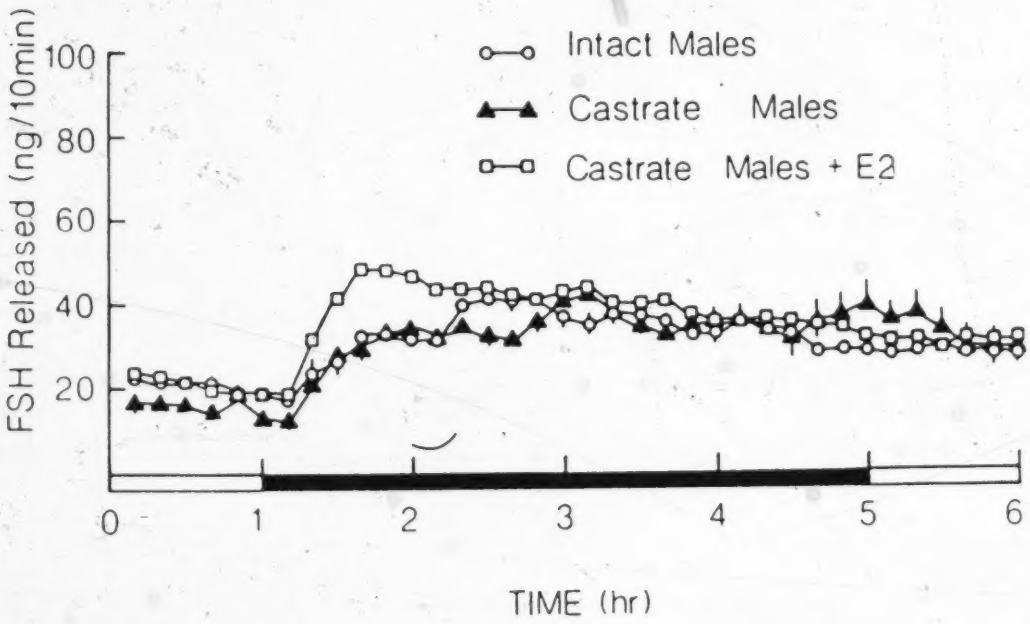
* Values represent the mean ± SEM (n=3). The 0 values in the Table were used for the pituitaries from intact and castrated males since these pituitaries did not respond to PMA infusion (cf. Figs. 3 and 6).

(Table 9) , illustrating the occurance of synergy. In contrast, the PMA- and ionomycin-activated mechanisms did not synergize in pituitaries obtained from castrated + E_2-treated males (cf. Table 9). The fact that synergistic responses were obtained from pituitaries of intact and castrated males indicates that the synergistic interaction between PMA and ionomycin activated mechanisms is not dependent on gonadal factors.

The experiments described above were repeated in the presence of cycloheximide to investigate the potential requirement of protein synthesis for the interactions between PMA and ionomycin. The results are shown in Fig. 18. Cycloheximide attenuated the responses from the pituitaries of the three treatment groups. The amounts of gonadotropins released were similar to those obtained during ionomycin infusions alone in the presence of cycloheximide ($p > 0.05$). Apparently, the dependency of PMA and ionomycin interactions on protein synthesis, reflects the association of PMA-stimulated mechanisms with de novo protein synthesis.

Fig. 18. Secretory profiles of LH and FSH obtained from pituitaries of intact, castrated and castrated + E_2-treated males in response to continuous infusion (4 h) of 10 uM ionomycin and 1 uM PMA simultaneously (indicated by the black bar) in KIRB medium in the presence of cycloheximide (n=3). Cycloheximide was present in the medium from the time of collection of the pituitaries and throughout the experiment. For further details see legend for Fig. 2.


Intact males
Castrate Males
Castrate Males + E2
LH Released (ng/10min)
100
80
60
40
20
1
2
3
4
5
6
TIME (hr)


Intact Males
Castrate Males
Castrate Males + E2
FSH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

IV. Experiments with Pharmacological Inhibitors of Protein Kinase C and Calmodulin.

The final series of experiments was designed to use pharmacological inhibitors of protein kinase C and calmodulin in an attempt to strengthen inferences on potential roles of PKC and calmodulin in the mechanism of gonadotropin secretion. Different chemical classes of protein kinase C and calmodulin inhibitors were tested including staurosporin (to inhibit PKC) and pimozide and W7 (to inhibit calmodulin).

One nM GnRH was infused to pituitaries of male rats in the presence of 10 nM staurosporin. As shown in Fig. 19, this concentration of staurosporin partially inhibited both the early and the late gonadotropin responses to GnRH, indicating that the compound, did not only inhibit PKC but it also inhibited calmodulin, i.e. it is not specific for PKC. This observation adds to the fact that staurosporin also inhibits the cyclic AMP-dependent protein kinase (PKA) (cf. Kamiya Biomedical Co. Manual) and cAMP was shown to be necessary for both the early and late phases of gonadotropin secretion (Bourne and Baldwin, 1987b). So the use of staurosporin to test the involvement of PKC in the GnRH-stimulated gonadotropin secretion would be nonspecific.

Pimozide as an inhibitor of the calcium-mobilization-activated mechanisms was tested in the tissue perifusion system. Pimozide did not dissolve readily in the perifusion medium, and it was partially precipitated when the medium was gassed with the oxygen and carbon dioxide mixture. Infusions of 1 nM GnRH to pituitaries obtained from male rats in the presence of pimozide caused partial inhibition of the early

and late LH secretory response to GnRH (Fig. 20). Given the fact that calcium mobilization-activated mechanisms (including calcium-calmodulin) are involved in the early and late responses (cf. Fig. 14), I expected pimozide to totally inhibit the early and partially inhibit the late LH responses to GnRH. The inability of pimozide to inhibit the ionomycin-induced LH secretion (Fig. 21) indicated that pimozide was not a potent specific inhibitor of calmodulin and that it may be also inhibiting PKC. This caused significant difficulties in data interpretation.

When W7 (100 uM) was tested in the tissue perifusion system as an inhibitor of the calcium-calmodulin system, the compound partially inhibited the early and late LH secretion from the anterior pituitaries of male rats in response to infusion of 1 nM GnRH (cf. Fig. 22). This was interpreted as a nonspecific inhibition.

In view of the nonspecificity of the PKC and calmodulin inhibitors tested, the experiments with these inhibitors were aborted.

Fig. 19. Secretory profile of LH obtained form pituitaries of intact males in response to continuous infusion (4 h) of 1 nM GnRH in KIRB medium in the presence of 10 nM staurosporin. As a control, refer to Fig. 8.

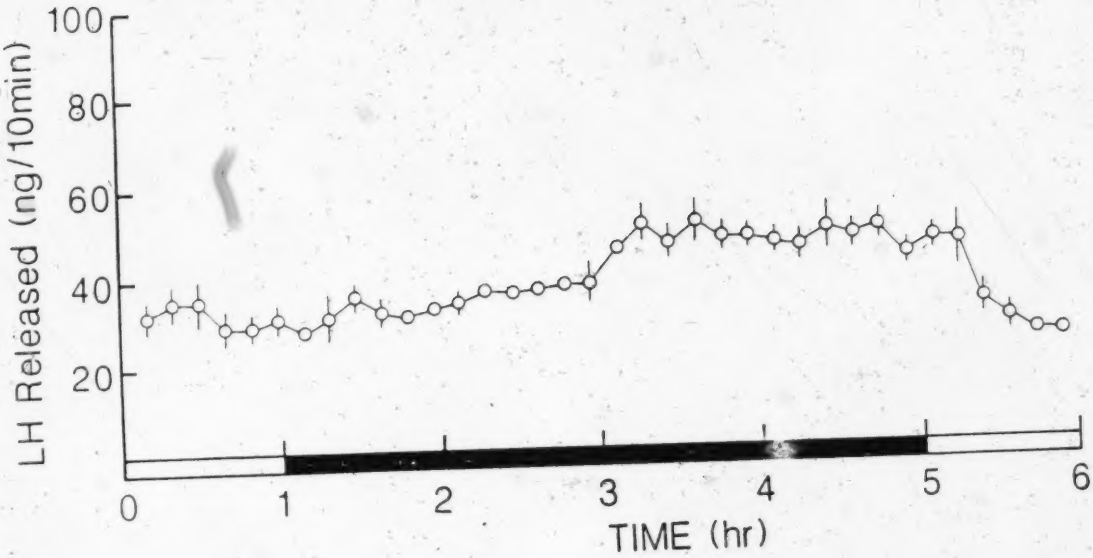

LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

Fig. 20. Secretory profile of LH obtained from pituitaries of intact males in response to continuous infusion (4 h) of 1 nM GnRH in KIRB medium in presence of 10 uM pimozide. As a control, refer to Fig. 8.

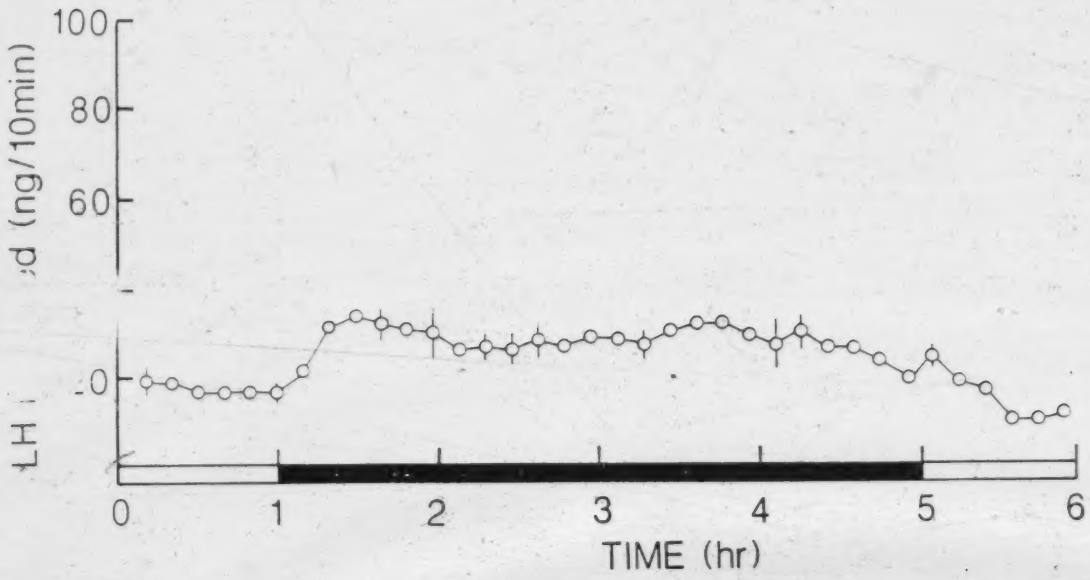

100
80
60
d (ng/10min)
LH
0
1
2
3
4
5
6
TIME (hr)

Fig. 21. Secretory profile of LH obtainted from pituitaries of intact males in response to continuous infusion (4 h) of 10 uM ionomycin in KIRB medium in presence of 10 uM pimozide. As a control, refer to Fig. 14.

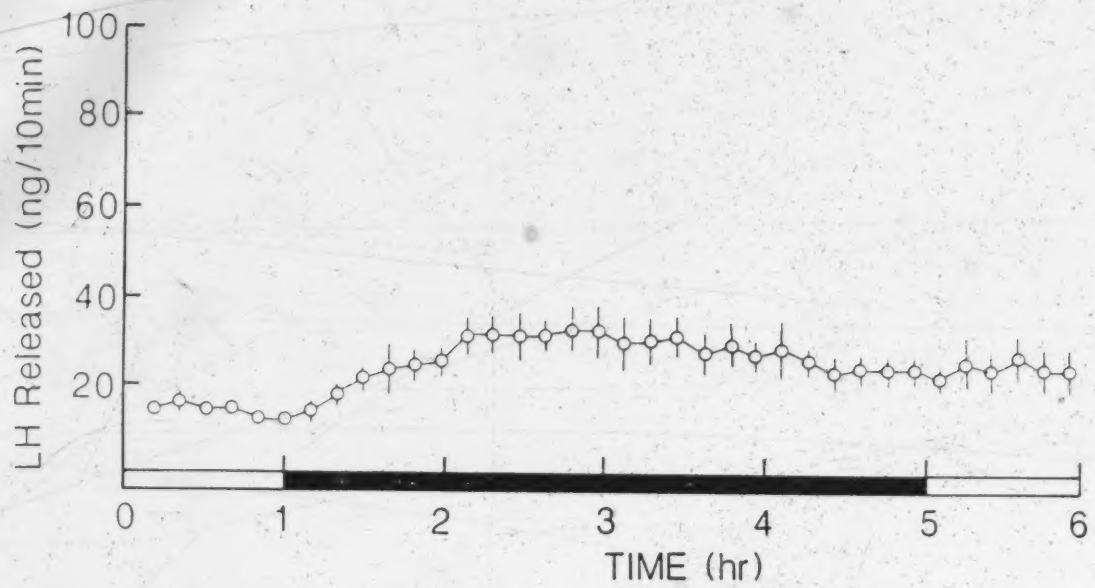
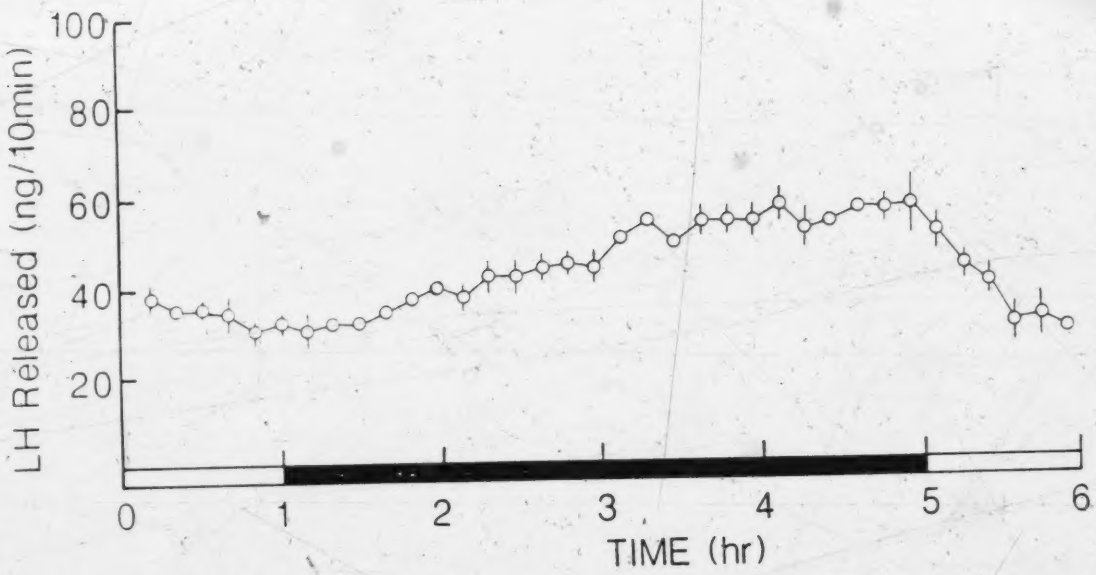

LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

Fig. 22. Secretory profile of LH obtained from pituitaries of intact males in response to continuous infusion (4 h) of 1 nM GnRH in KIRB medium in the presence of 100 uM W7. As a control, refer to Fig. 8.


LH Released (ng/10min)
100
80
60
40
20
0
1
2
3
4
5
6
TIME (hr)

CHAPTER IV

DISCUSSION

The present study was undertaken to investigate the signal transduction roles of the two branches of the calcium messenger system during the secretion of LH and FSH from male anterior pituitaries. A perifusion system was chosen for the fulfillment of the research objectives, because it facilitates the sequential modification of medium without tissue manipulations, and circumvents an inherent problem of static systems in that it prevents the accumulation of proteolytic enzymes, metabolic products and anterior pituitary hormones and their possible feedback and/or destructive effects. Furthermore, stable basal release rates of the gonadotropins are obtained prior to the administration of secretagogues, furnishing an appropriate control to evaluate the release obtained in response to these secretagogues i.e. each perifusion chamber effectively serves as its own control. Additionally, the system is simple in design, manipulable and more importantly provides responses which are highly reproducible (cf. Appendix 4).

A tissue perifusion system was used instead of a dispersed pituitary cell perifusion system, because the pituitary tissue perifusion system duplicates in vivo secretory profiles and phenomena, while dispersed pituitary cells do not. Basically, the kinetics of the in vitro biphasic release of LH obtained during continuous infusions of GnRH to pituitary tissue blocks of females in perifusion systems (Bourne and Baldwin, 1980; Waring and Turgeon, 1980; Baldwin et al., 1983; Bourne and Baldwin,

1987a&b; Das et al., 1989), closely resemble the kinetics of the secretory responses obtained in vivo from female rats (Blake, 1976; 1978a). In contrast, the biphasic responses obtained from the perifusion systems using dispersed pituitary cells (Hopkins, 1977; Naor et al., 1982; Catt et al., 1983; Hansen et al., 1987) bear no resemblence temporally to the secretory profiles obtained in vivo. Furthermore, the cells did not exhibit the self-priming effect when GnRH pulses were administered (Naor et al., 1982; Catt et al., 1983; Chang et al., 1987; 1988), while the phenomenon was apparent when tissue blocks were used (Edwardson and Gilbert, 1975; Bourne and Baldwin, 1980; Waring and Turgeon, 1980; Moll and Rosenfield, 1984). Taken together, these observations provide some confidence that the pituitary tissue perifusion system used in the present study may respond similarly to in vivo responses, while dispersed cells may not.

The basic approach of this study was to use pharmacological agents to mobilize calcium and/or activate protein kinase C. Ionomycin (a calcium ionophore) intercalates in both plasma membranes and intracellular membranes forming a conduit which permits the movement of calcium down its concentration gradients. The end result is an increase in cytoplasmic calcium concentration.

The increased cytoplasmic calcium concentration in turn activates a number of systems. These include the calcium-calmodulin system and its dependent enzymes as well as other calcium-dependent processes such as cyclic nucleotide metabolism, arachidonic acid metabolism and control of microtubule assembly. Thus, calcium mobilization could conceivably activate other processes apart from those activated by calcium calmodulin.

Ionomycin was chosen as the calcium mobilizing agent instead of other ionophores, because it is more specific for calcium (Liu and Herman, 1978), and is most effective in stimulation of LH secretion (Chang et al., 1986).

In spite of the fact that diacylglycerols are the physiological activators of PKC, PMA was used in this study to activate the enzyme because DAG were recently shown to stimulate the turnover of inositol phosphates in the anterior pituitary (Huckle and Conn, 1987), raising the possibility that exogenous DAG might increase the intracellular concentration of IP_3. Since IP_3 is known to mobilize calcium from intracellular stores (Berridge and Irvine, 1984), its production by DAG could conceivably increase cytoplasmic calcium and activate the calcium-mobilization-activated system(s). This in turn would pose significant problems with data interpretation. Serendipitously, it seems that PMA, does not suffer from this potential disadvantage, since it does not directly increase IP production (Huckle and Conn, 1987), or affect phospholipid turnover in enriched gonadotrophs (Andrews and Conn, 1986).

Unfortunately, specific inhibitors of protein kinase C or calmodulin are not available. This fact coupled with the potential non-specific effects of ionomycin and PMA limits data interpretation. While PMA does in fact activate PKC in anterior pituitaries (Chang et al., 1987) and ionomycin does activate the calcium-calmodulin system, (Rasmussen and Barrett, 1984), the potential nonspecific effects of these secreatagogues can not be excluded and should be kept in mind.

I. Estradiol-Dependency of PMA-Stimulated Gonadotropin Secretion from the Anterior Pituitaries of Male and Female rats.

In the initial study, the PMA-induced secretion of gonadotropins was investigated with respect to a dependency on estradiol. Pituitaries obtained from diestrous II and OVX + E_2-treated animals were responsive to PMA, whereas intact males as well as gonadectomized males and females were not responsive. Interestingly, implantation of estradiol capsules in the castrated males resulted in their pituitaries becoming responsive to PMA. To the author's knowledge, this is the first report which clearly demonstrates a sex-difference in the efficacy of PMA to directly induce gonadotropin secretion. Moreover, it appears that this sex-difference in the efficacy of PMA to act as a direct secretagogue of gonadotropin secretion is dependent on estradiol.

Within this framework of a dependency of PMA-induced gonadotropin secretion on estradiol, the present results also indicate at least two concentration-dependent effects of the steroid in females. In the first instance, the levels of circulating estradiol appear to be an important determinant of the time of onset of PMA-stimulated secretion of gonadotropins. When estradiol levels were maintained at proestrus concentrations (60-75 pg/ml) with the implants (cf. Legan et al., 1975 ; Baldwin et al., 1983 and Appendix 1), PMA-stimulated secretion from pituitaries of OVX +E_2-treated females was manifest within 30 minutes. In contrast, the secretion from pituitaries of diestrous II females (with E_2 levels 40 pg/mL, cf. Appendix 1) did not become apparent until after 60 minutes. Secondly, the magnitude of the secretory responses to PMA also appears to be dependent on the concentration of estradiol, since the

responses from pituitareis of OVX + E_2-treated females were greater than the responses obtained from pituitares of diestrous II females. Taken together, these observations indicate that estradiol increased the responsiveness of the PMA-mediated secretory mechanisms in gonadotrophs in a dose dependent manner.

The fact that estradiol induces an enhanced responsiveness to GnRH is well established (Aiyer et al., 1974; Castro-Vazquez and McCann, 1975; Waring and Turgeon, 1980; Turgeon and Waring, 1981; Baldwin et al., 1983; Frawley and Neill, 1984; Moll and Rosenfield, 1984). In spite of the extensive documentation of these effects of estradiol, the molecular mechanisms involved in the estradiol-induced enhanced responsiveness of gonadotrophs to GnRH remain enigmatic. However, a study by Baldwin et al. (1983) had indicated that the increased responsiveness of the gonadotrophs brought about by estradiol occurs during the second phase of the biphasic secretion of LH from pituitaries obtained from diestrous II female rats. Since PKC-stimulated gonadotropin secretion occurs in the second phase (Das et al., 1989), this observation by Baldwin et al. (1983) is consistent with the present results, and a report by Liu and Jackson (1987). These observations suggest that at least one basis for the estradiol-induced increased responsiveness of gonadotrophs to GnRH appears to involve the secretion of LH which is directly stimulated by PMA. If the effects of PMA can be attributed to its activation of protein kinase C, then one underlying basis for the estradiol-induced increased responsiveness of gonadotrophs to GnRH is manifest in the ability of protein kinase C to stimulate gonadotropin secretion in the absence of calcium mobilization. Since protein kinase C interacts with calcium-mobilizating mechanisms to mediate the extracellular

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calcium-dependent phase II component of gonadotropin secretion from female pituitaries (Das et al., 1989), this component may also be involved in the estradiol-induced increased responsiveness of gonadotrophs to GnRH.

On the surface, the inability of pituitaries from intact or orchidectomized males to respond to PMA with an increased secretion of LH and FSH could be construed to suggest that protein kinase C does not normally play a role in GnRH-stimulated gonadotropin secretion in pituitaries of male rats. However, evidence against this possibility was obtained from other experiments (cf. table 9) which demonstrated the manifestation of synergistic interactions between ionomycin and PMA from pituitaries of intact or castrated males. These observations suggest that the activation of calcium mobilization is mandatory for the expression of an involvement of PMA-activated mechanisms as a mediator of GnRH-stimulated gonadotropin secretion from pituitaries of intact or castrated males.

The fact that perifused pituitaries obtained from castrated males were capable of responding to PMA after exposure to estradiol, indicates that the basic mechanisms involved in PMA-stimulated gonadotropin secretion (in the absence of calcium mobilization) could also be induced in castrated males.

The mechanisms by which estradiol affects the activation of protein kinase C to directly induce the secretion of LH are unknown. However, it seems reasonable to suggest that estradiol conceivably causes: i) increased synthesis and/or activity of protein kinase C (cf. Drouva et al., 1990), ii) increased synthesis and/or activity of proteins which are involved in the secretory mechanisms and serve as substrates for protein

kinase C, iii) increased synthesis and/or activity of cytoskeletal or contractile protein(s) that facilitates the mobilization of PKC from the cytosol to the plasma membrane (Kamel and Krey, 1983; Goldstein et al., 1975), and/or iv) increased synthesis and/or activity of proteins which could neutralize the actions of the endogenous inhibitors of protein kinase C (cf. Kraft and Anderson, 1983; Turgeon et al., 1984; Naor et al., 1985b). If the latter possibility does indeed occur, it also necessitates the postulate that calcium mobilization can neutralize the inhibitors in pituitaries of males, permitting the synergistic interactions between calcium-mobilization-activated mechanisms and protein kinase C. Obviously, the hypotheses enumerated above are not mutually exclusive and the effects of estradiol may actually reflect a composite of all or some of these possibilities.

In contrast to the present results obtained from perifused intact male pituitary tissue blocks, other reports had demonstrated that dispersed male anterior pituitary gonadotrophs respond to PMA with an increased secretion of LH (Smith and Vale, 1980; 1981). These results with cells were confirmed in the present study (cf. Fig. 7). Based on the results obtained from pituitaries of castrated + E_2-treated females (cf. Fig. 6), it seemed plausible that the ability of dispersed male cells to respond to PMA may be due to the effects of estradiol in the supplementary sera added to the culture medium. Thus, the responses obtained from dispersed cells obtained from anterior pituitaries of male rats cultured in Ham's F10 supplemented with normal male rat serum could be due to the estradiol content of the male rat serum (although very low, cf. Appendix 1) or due to the possible estrogenic activity of phenol red contained in the culture

medium (cf. Drouva et al., 1990).

II. Induction of an Extracellular Calcium-Independent Component of Gonadotropin Secretion from the Anterior Pituitaries of Male Rats.

Previous reports from this laboratory had demonstrated the manifestation of a delayed onset (30-60 min), extracellular calcium-independent component of gonadotropin secretion from pituitaries of diestrous II female but not male pituitaries (Bourne and Baldwin, 1980; Bourne, 1988; Bourne et al., 1988). This response was shown to be dependent on estradiol (Bourne et al., 1988), and appeared to be duplicated (at least in part) by PMA (Bourne et al., 1989). Since, in the first study, the PMA-stimulated gonadotropin secretion from male pituitaries was shown to be an estradiol-dependent phenomenon, the next logical question to be investigated was whether estradiol could induce a GnRH and/or PMA-stimulated, extracellular calcium-independent component of gonadotropin secretion from pituitaries of castrated males.

The responses to GnRH in calcium-free medium from pituitaries of intact, castrated and castrated + E_2-treated males were temporally similar to those in the KIRB medium but much lower in amounts, demonstrating the importance of extracellular calcium in maintaining the normal levels of gonadotropin secretion from male pituitaries. It was also interesting to note that, similar to the situation in KIRB medium, LH secretion in calcium-free medium was higher than FSH secretion. This indicates that the absence of calcium in the extracellular medium, does not interfere with the mechanisms regulating the LH/FSH ratio which is critical in maintaining normal gonadal function (Yen et al., 1970; Rebar, et al.,

1976; Yen, 1980).

Surprisingly, however, during the course of these experiments, pituitaries obtained from both intact and castrated males responded rapidly to GnRH in the absence of extracellular calcium (cf. Fig. 9). These low, instantaneous, extracellular calcium-independent responses conflicted with previous reports (Bourne, 1988; Bourne et al., 1988) which demonstrated the absence of an extracellular calcium-independent secretion of LH from male pituitaries. After repenting the experiments several times (to ensure the reproducibility of the results), a careful review of all experimental data indicated that the experiments in which pituitaries of males did not respond to GnRH were performed in the fall and winter, whereas the experiments with responsive pituitaries were undertaken in late spring and summer. These observations, coupled with the demonstration of rhythms for a number of signal transduction mechanisms (Takahashi and Zatz, 1982), made it tempting to speculate that pituitaries of male rats might be exhibiting a rhythm in which changes occur approximately every 12 months, and are observed even under constant environmental conditions, i.e. a free-running circannual rhythm (Takahashi and Zatz, 1982). If this speculation is correct, the rhythm would be associated with changes in the roles and requirements of various components of signal transduction mechanisms. At least one of these changes would be manifest during spring and summer by the induction of a rapid onset, low extracellular calcium-independent secretion of LH and FSH, a secretory component that is normally absent during the remainder of the year. It is also conceivable that the different responses could be due to changes in the hormonal supplements (especially estradiol) in the

rat food. Obviously, future studies over a period of several years will be required to test these hypotheses.

Apparently, the intracellular mechanisms mediating the GnRH-stimulated extracellular calcium-independent release of gonadotropins from pituitaries of intact and castrated males differ from those which are operative in the pituitaries of intact females. In the first instance, responses from the pituitaries of males have a rapid onset (cf. Fig 9), while the responses from pituitaries of females have a delayed onset (Bourne and Baldwin, 1980; Bourne 1988; Bourne et al., 1989). Secondly, the responses from pituitaries of females which are mimicked by PMA (Bourne et al., 1989) are completely dependent on protein synthesis (Bourne and Baldwin, 1980; Bourne et al., 1989), whereas the rapid onset responses from pituitaries of males are not. These observations suggest that the rapid onset responses from pituitaries of males are not mediated by PKC as might be the the case with females (Bourne et al., 1989), but are similar to the dispersed pituitary cells' responses which are immediate (Catt et al., 1983), and appear to be mediated by the mobilization of intracellular calcium (Naor et al., 1988). If this, is indeed the case, then the low, rapid onset extracellular calcium-independent secretion of gonadotropins from male pituitaries might be due to the mobilization of intracellular calcium.

In this regard, it is interesting to note that phospholipase C activation appears to occur in the absence of extracellular calcium (Naor et al., 1985a, Huckle and Conn, 1987; Andrews and Conn, 1986). Consequently, the GnRH-stimulated, rapid onset, extracellular calcium-independent component of gonadotropin secretion from pituitaries

of males could be due to the hydrolysis of phosphatidyl inositol bisphosphate by phospholipase C into diacylglycerol and inositol 1,4,5 trisphosphate (IP_3). The fact that IP_3 rapidly mobilizes calcium from intracellular stores (Berridge, 1984), makes it conceivable that this component of GnRH-stimulated gonadotropin secretion might indeed be due to the mobilization of intracellular calcium.

Additionally, the results of the present study demonstrate that estradiol enhances GnRH-stimulated gonadotropin secretion from pituitaries of castrated males perifused with calcium-free medium. Interestingly, the enhanced secretion was delayed, becoming evident approximately 80 minutes after GnRH administration (cf. Fig. 10). Moreover, this estradiol-induced enhanced response was inhibited by cycloheximide suggesting a requirement for de novo protein synthesis. These observations suggest that the estradiol-enhanced response may be due to the same mechanisms which are operative in female gonadotrophs. Furthermore, the fact that cycloheximide inhibited the enhanced component of GnRH-stimulated gonadotropin secretion from pituitaries of castrated + E_2-treated males reducing it to the low levels of the rapid onset secretion obtained from pituitaries of intact and castrated males, suggest that the GnRH-stimulated extracellular calcium-independent gonadotropin secretion from pituitaries of castrated + E_2-treated males might actually represent a composite response. Thus, the response appears to consist of an initial, rapid onset, partially protein synthesis-dependent component that may be due to the mobilization of intracellular calcium, and a delayed response that is completely dependent on de novo protein synthesis.

Unlike GnRH, PMA, stimulated gonadotropin secretion from the pituitaries of castrated + E_2-treated males only. Furthermore, the PMA-induced response was characterized by exhibiting a delayed onset (approximately 40-50 min), and by being completely dependent on de novo protein synthesis. These results extend the observation of the first study and indicate that estradiol also enhances gonadotropin secretion from pituitaries of castrated males perifused with calcium-free medium by affecting the PMA-stimulated mechanisms.

The estradiol-enhanced GnRH-stimulated secretion is similar to the PMA-induced secretion in that both have a delayed onset and are dependent on de novo protein synthesis. This in turn suggests that common cellular mechanisms might be operative. If the PMA-induced secretion is in fact due to the activation of PKC, then it seems likely that the GnRH-stimulated estradiol-enhanced component might be mediated by PKC. In short, the GnRH-stimulated gonadotropin secretion obtained from castrated + E_2-treated male pituitaries perifused with calcium-free medium appears to consist of a rapid onset component mediated by the mobilization of intracellular calcium, and a protein synthesis-dependent, delayed component which might be mediated by PMA-activated mechanisms, presumably PKC.

III. Interactions Between the Secretory Mechanisms Activated by Ionomycin and PMA in the Anterior Pituitaries of Male Rats.

The final study was designed to investigate potential interactions between ionomycin- and PMA-stimulated mechanisms in anterior pituitaries of male rats, and the effects of estradiol on these interactions. The

results of this study confirm earlier reports (Conn et al., 1980; Chang et al., 1986; Das et al., 1989) that ionomycin stimulates gonadotropin secretion from the anterior pituitary. The ionomycin-induced LH and FSH secretions from pituitaries of intact and castrated males were qualitatively (Fig. 14) and quantitatively similar (cf. Table 9), indicating that the short-term absence (72 h) of testicular factors (steroids and/or peptides) does not affect gonadotropin secretion obtained in response to calcium mobilization. In contrast, an enhanced response was obtained from pituitaries of castrated + E_2-treated males.

The mechanisms through which estradiol enhances the secretory response to the calcium mobilizing agent are not known. However, the results from this study suggest that it is a protein synthesis-dependent phenomenon, since only the enhanced component of the response from pituitaries of castrated + E_2-treated males was inhibited by cycloheximide. The protein synthesis inhibitor did not affect the ionomycin-induced gonadotropin secretion from pituitaries of intact or castrated males or a corresponding component from pituitaries of castrated + E_2-treated males (cf. Fig. 16). As a result, the effects of estradiol on the ionomycin-induced secretion may be due to i) increased synthesis of gonadotropins (Tang, 1980), ii) increased availability of gonadotropins for secretion, since it was reported that calcium mobilization results in the secretion of gonadotropins from a "readily available pool" (Hoff et al., 1977), or iii) Increased synthesis of the effector proteins that mediate secretion e.g. the calcium-mobilization-dependent enzymes. It should be noted that these possibilities are not mutually exclusive, and the effects of estradiol could be the result of some or all of the above.

In addition to enhancing gonadotropin secretion from pituitaries of castrated + E_2-treated males, there was also a decline in gonadotropin secretion from these pituitaries after two hours of ionomycin or GnRH infusions (cf. Figs. 8 & 14). It is noteworthy that after approximately two hours, ionomycin and GnRH induced a decline in the rate of secretion, while PMA did not. This suggests that the GnRH-induced decline in the rate of secretion probably involves mechanisms associated with calcium-mobilization. It is also interesting that this phenomenon was not apparent from pituitaries of castrated or intact males, and the secretion from the pituitaries of castrated + E_2-treated males decreased to the levels obtained from pituitaries of intact and castrated males. These observations suggest that in this instance the decline in secretion is only associated with the estradiol-enhanced component of gonadotropin secretion. Furthermore, the fact that the estradiol-enhanced secretion is dependent on de novo protein synthesis, suggests that the decline in gonadotropin secretion might be related to the newly synthesized protein(s) induced by estradiol. As such, the decline in secretion observed from pituitaries of from castrated + E_2-treated males may be due to a relatively rapid turnover rate for the protein(s) whose synthesis is stimulated by estradiol, and/or the fact that the rate of release exceeds the rate at which the "releasable" gonadotropins are made available for secretion. This second possibility is based on a report that the initial rapid onset of gonadotropin secretion caused by calcium-mobilization is due to the release of a readily available pool of gonadotropins (Tang, 1980; Hoff et al., 1977). Obviously, both possibilities can be combined resulting in a situation where the availability of the "releasable"

gonadotropins may require newly synthesized enzymes which have a relatively rapid turnover rate.

Infusions of PMA alone to intact or castrated male anterior pituitaries, did not stimulate gonadotropin secretion (cf. Figs. 2 & 6), while infusions of ionomycin alone caused a rapid onset of gonadotropin secretion. However, simultaneous infusions of both compounds to pituitaries of intact or castrated males, resulted in synergy between these secretagogues. This is illustrated by the fact that the total amounts of hormones secreted in response to the simultaneous infusions of PMA and ionomycin was greater than the algebric sum of the hormones secreted in response to either of the secretagogues alone (cf. Table 9). Thus, although PMA did not stimulate gonadotropin secretion from intact or castrated male pituitaries, the phorbol ester activated mechanisms which interacted with those activated by ionomycin. This may be indicative of the fact that calcium mobilization is normally a prerequisite for the manifestation of a role for protein kinase C as a mediator of gonadotropin secretion from pituitaries of male rats.

The release of gonadotropins in response to simultaneous infusions of PMA and ionomycin to pituitaries obtained from intact or castrated males were quantitatively and qualitatively similar indicating that, at least in the short term (3 days) castrated males, the synergistic interactions between these secretagogues are not affected by testicular factors.

The cellular mechanisms underlying the synergistic interactions are presently unknown, but appear to be dependent on protein synthesis. The synergy may be due to some or all of the following possibilities. First, PMA and/or ionomycin may activate other signal transduction systems that

play a role in gonadotropin secretion (e.g. the cAMP-adenylate cyclase system and/or the arachidonic acid-lipooxygenase pathway). In this regard, it is interesting to note that the secretagogues stimulated cAMP production in a dose dependent manner in male pituitaries (Bourne, G.A., unpublished data). Obviously, the recruitment of other mediators could result in an increased response. Secondly, ionomycin may result in the activation of certain proteins associated with the secretory mechanisms, while PMA may activate others. The combination of these agents together would then result in the simultaneous activation of all of these proteins, culminating with the synergistic response. For example, in platelets, thrombin is known to increase the phosphorylation of both a 40 KDa and a 20 KDa protein (Lyons and Alberton, 1979). The 40 KDa protein appears to be a specific substrate for protein kinase C (Kaibuchi et al., 1982; 1983), while the 20 KDa protein is normally phosphorylated by calcium calmodulin (activated by the ionomycin-induced increases in cytoplasmic calcium concentration) (Kaibuchi et al., 1982; 1983). When PMA and ionomycin are administered together, both proteins are phosphorylated resulting in a synergistic response. Additionally, it is also conceivable that the activation of the calcium-mobilization system could counteract the effects of the endogenous inhibitors of protein kinase C thus facilitating the expression of the full activity of the PMA-stimulated mechanisms. Another possibility is based on the ability of the phorbol ester to sensitize effector mechanisms to the actions of calcium, as described in other systems (Whitefield et al., 1973; Knight and Barker, 1983; Knight and Scrutton, 1983). In thymic lymphocytes, the phorbol esters were suggested to sensitize the effector system (in this case DNA

synthesis) to the action of calcium (Whitefield et al., 1973), while in adrenal medullary cells DAG apparently altered the responsiveness of the exocytosis process for calcium (Knight and Barker, 1983). Similar conclusions were drawn from a study by Knight and Scrutton (1983) on permeabilized blood platelets where thrombin and 1-oleoyl, 2-acetyl glycerol were shown to increase the sensitivity of the secretory cells to calcium. The increased sensitivity to calcium would then result in an increased secretory response.

Surprisingly, synergistic interactions were not apparent from pituitaries obtained from castrated + E_2-treated male rats. The algebric sum of the hormones secreted in response to either PMA or ionomycin alone was not significantly different from the amounts secreted in response to both secretagogues infused simultaneously (cf. Table 9). Since the synergistic interactions of PMA and ionomycin-activated mechanisms are protein synthesis-dependent, and estradiol enhances the responses to both secretagogues by protein synthesis dependent mechanisms, it seems highly probable that the absence of synergism in gonadotrophs obtained from castrated + E_2-treated males is due to the fact that estradiol had already induced the synthesis of the protein(s) which play a role in synergism. Obviously, future experiments will be required to clarify this issue.

SUMMARY

Using an anterior pituitary tissue perifusion system which duplicates *in vivo* gonadotropin secretory profiles and phenomena, I have demonstrated that PMA-stimulated gonadotropin secretion (in the absence of calcium mobilization) is an estradiol and protein synthesis-dependent phenomenon. Moreover, in females, the concentration of estradiol appears to determine both the magnitude and the time of onset of the PMA-induced response, forming at least one basis for the increased responsiveness to GnRH normally seen in female gonadotrophs as a result of the effects of estradiol.

Pituitaries of intact and castrated males perifused with calcium-free medium responded to GnRH with a low, rapid component of gonadotropin secretion which might be mediated by calcium-mobilization mechanisms. In contrast, a composite secretory response was obtained from pituitaries of castrated + estradiol-treated males. The response included the calcium mobilization-mediated, low, rapid onset protein synthesis-independent component, plus a delayed secretory component (about 70 min) which was dependent on estradiol and protein synthesis, and was hypothesized to be mediated by protein kinase C.

The occurrence of protein synthesis dependent synergistic interactions between ionomycin and PMA (even though PMA alone was an ineffective secretagogue) in pituitaries obtained from castrated and intact males, suggests a role for PMA-activated mechanisms (presumabely PKC) during GnRH-stimulated secretion. However, the demonstration of a definitive

role for the enzyme will be dependent on the availability of specific and potent PKC inhibitors. In addition to the absence of synergistic interactions between PMA and ionomycin, the enhanced gonadotropin secretion after exposure to estradiol showed a decline in the rate of secretion despite the continued presence of ionomycin or GnRH, suggesting that the decline of secretion in both cases might be mediated by similar underlying mechanisms.

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APPENDIX 1. Postimplantation serum estradiol concentrations in female and male rats used in this study (weight of the rats was 250-300 g).

Group	Serum concentration at the time of sacrifice*.
Intact males	11.2 ± 1.7 pg/mL
Intact females (DII)	38.4 ± 3.5 pg/mL
Castrated males	9.6 ± 2 pg/mL
Ovariectomized rats	< 5 pg/mL
Castrated + E_2-treated males	113 ± 8 pg/mL
Ovariectomized + E_2-treated rats	65.7 ± 11.4 pg/mL

* Values represent the mean ± SEM (n=6).

N.B.

The same estradiol implants were used for males and females (cf. Materials and Methods).

APPENDIX 2. Composition of Kreb's Improved Ringer I Bicarbonate (KIRB) Medium.

80 parts of 0.154 M sodium chloride

4 parts of 0.154 M potassium chloride

3 parts of 0.154 M calcium chloride

1 part of 0.154 M potassium monobasic phosphate

1 part of 0.154 M magnesium sulfate

21 parts of 1.3 g % sodium bicarbonate

4 parts of 0.16 M sodium pyruvate

7 parts of 0.05 M sodium fumarate

4 parts of 0.16 M sodium glutamate

5 parts of 0.30 M glucose B(D+)

2.68 parts of MEM amino acid stock (as supplied by Flow Laboratories, McLean, VA, cf. Materials and Methods).

1.34 parts of MEM vitamins stock (as supplied by Flow Laboratories, McLean, VA, cf. Materials and Methods).

APPENDIX 3. Data and log-logit plots for representative radioimmunoassays for LH and FSH.

(i) LH.

Percent of total bound = 0.32

Mean counts

Concs.	Counts	Counts	Counts	Counts	Counts	Mean Counts
Totals	9289	9355	9227	9014	8992	9175
NSB	154	107	158	112	123	131
Bo	2988	2995	3082	3137	3140	3068

Logits

Concs	Counts	Counts	Counts	Mean Counts	Percent Bound	Logit
0.0625 ng	2770	2614	2715	2700	0.874	1.94
0.125 ng	2442	2339	2399	2393	0.770	1.21
0.250 ng	1997	1921	2009	1976	0.628	0.52
0.500 ng	1355	1410	1415	1393	0.430	-0.28
1.000 ng	885	932	868	895	0.260	-1.05
2.000 ng	536	576	593	568	0.149	-1.47

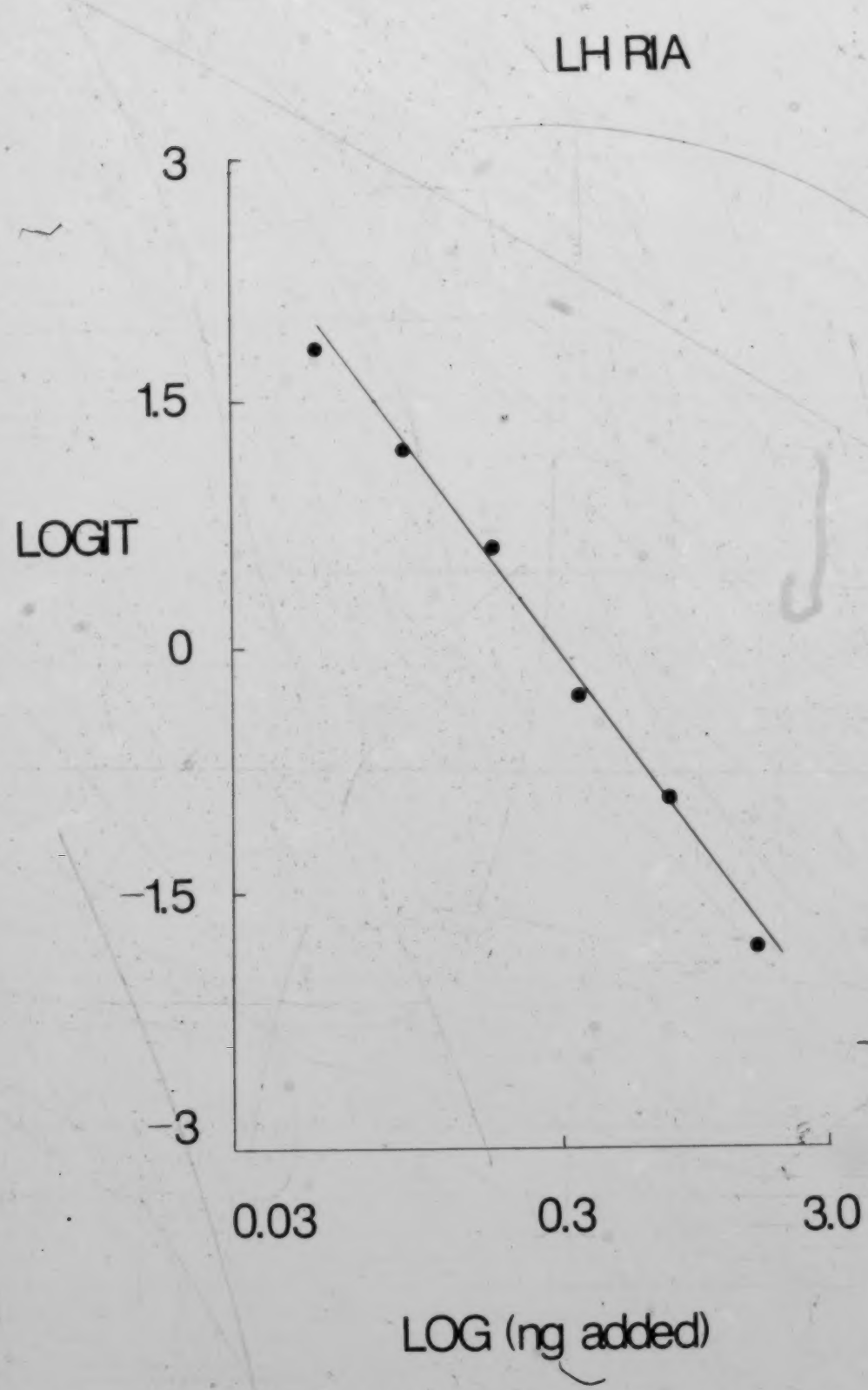
Unweighted regression analysis for the standard curve

Correlation coefficient = 0.9996
Y intercept = % - 1.0133
Slope = - 2.4668

Weighted regression analysis for the standard curve

Y intercept = % - 1.0186
Slope = - 2.4861

Percent bound	Concentration of LH
10%	2.98
50%	0.39
90%	0.05


LH RIA
3
1.5
LOGIT
0
-1.5
-3
0.03
0.3
3.0
LOG (ng added)

(ii) FSH

Percent of total bound = 0.33

Mean counts

Concs.	Counts	Counts	Counts	Counts	Counts	Mean Counts
Totals	10140	10036	10090	10065	10110	10088
NSB	85	66	81	80	76	78
Bo	3480	3318	3356	3348	3260	3352

Logits

Concs.	Counts	Counts	Counts	Mean Counts	Percent bound	Logit
0.3125 ng	3089	3124	3319	3137	0.934	2.65
0.6250 ng	2839	2896	2972	2902	0.863	1.84
1.2500 ng	2512	2527	2534	2524	0.747	1.08
2.500 ng	1986	2056	1927	1990	0.584	0.34
5.000 ng	1358	1347	1303	1336	0.384	-0.47
10.00 ng	857	810	812	826	0.229	-1.22

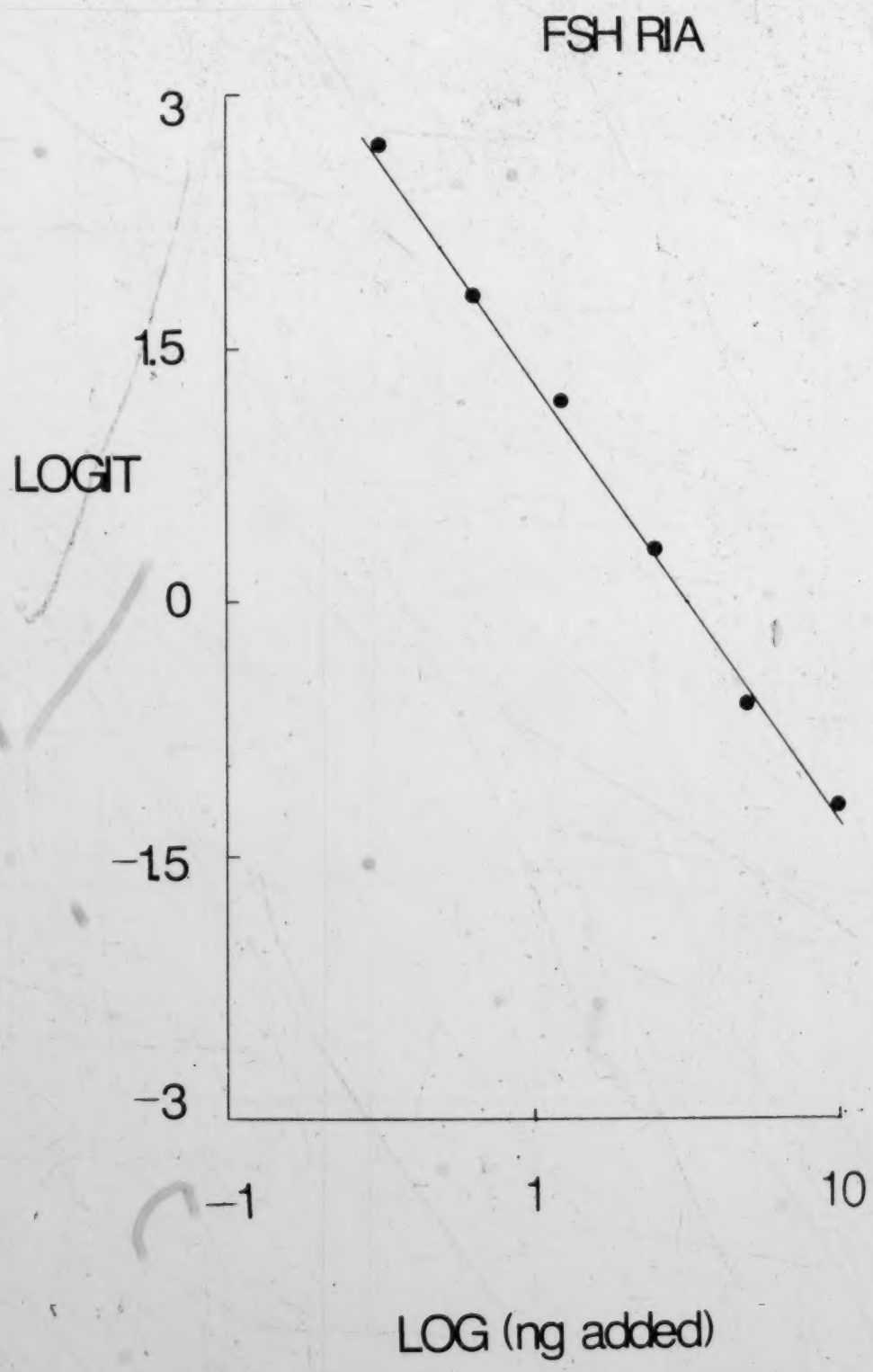
Unweighted regression analysis for the standard curve

Correlation coefficient = 0.9998
Y intercept = 1.3381
Slope = -2.5635

Weighted regression analysis

Y intercept = 1.3363
Slope = -2.5599

Percent Bound	Concentration of FSH
10%	= 24.01
50%	= 3.33
90%	= 0.46


FSH RIA
3
1.5
LOGIT
0
-1.5
-3
-1
1
10
LOG (ng added)

APPENDIX 4. Reproducibility of the responses obtained from pituitaries of diestrous II females in response to continuous infusions (4 h) of 10 uM ionomycin in KIRB medium. Dates of conduction of the experiments are from top to bottom:

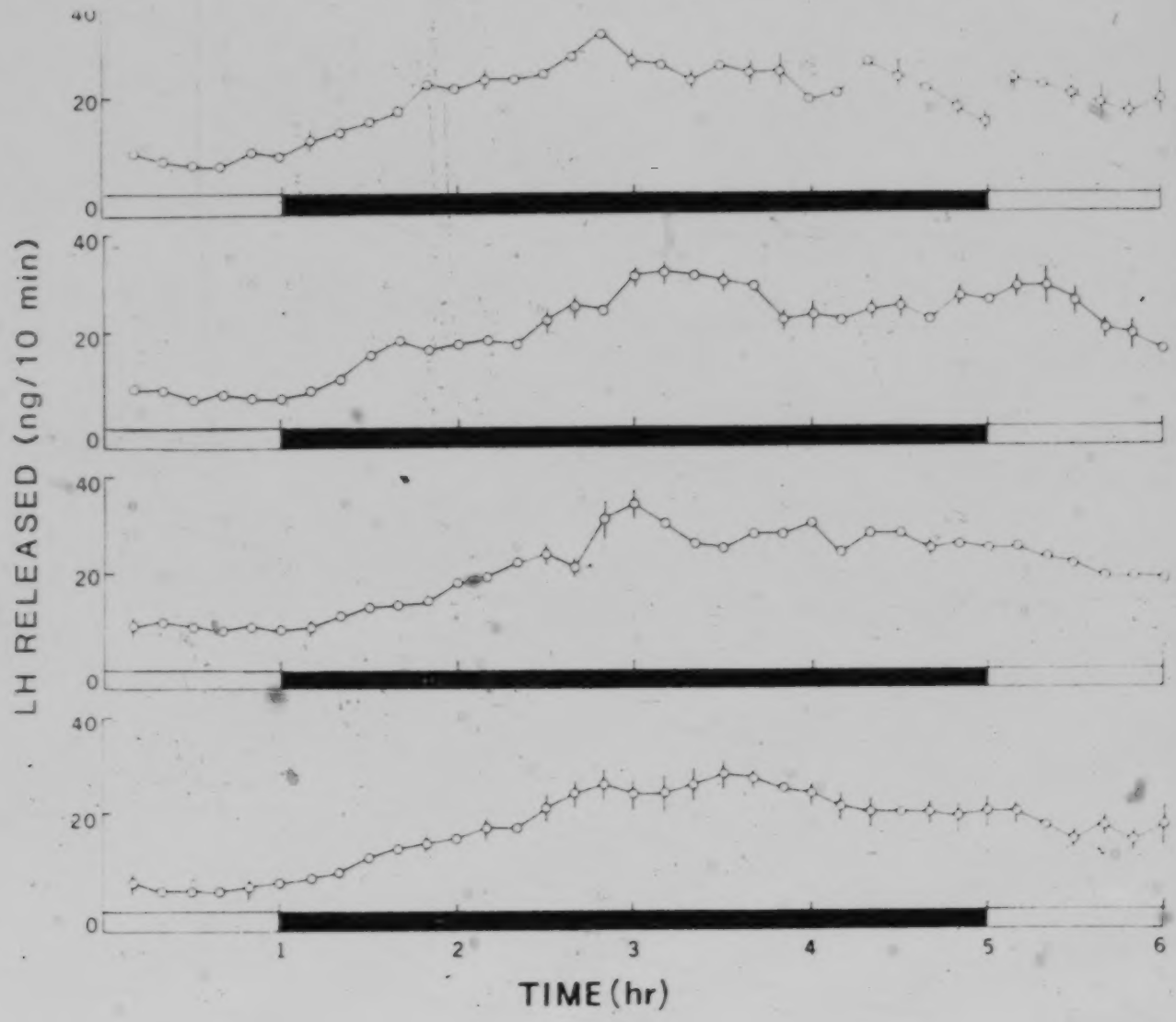
Jan. 8, 1990.

May 25, 1989.

Apr. 20, 1989.

Feb. 12, 1988.

N.B. Results courtesy of Dr. S. Das.


LH RELEASED (ng/10 min)
40
20
0
1
2
3
4
5
6
TIME (hr)